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The Carbohydrate Nutrition of Tomato Roots

II. The Mechanism of Sucrose Absorption by Excised Roots

BY

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With six Figures in the Text

INTRODUCTION

DORMER and Street (1949) have shown that excised tomato roots are able to utilize sucrose at a much greater rate than either dextrose, laevulose, or an equimolecular mixture of the two and have tentatively advanced the hypothesis that the mechanism of sucrose utilization involves the operation of a specific sucrose phosphorylase at the surface of the absorbing cells. The present investigation was undertaken to test this hypothesis and has involved a study of the effect of selected factors on the carbohydrate uptake of the roots.

GENERAL EXPERIMENTAL PROCEDURE

The general culture technique employed in the establishment and maintenance of our excised root cultures has been previously described (Dormer and Street, 1949). A clone of excised tomato roots (Best-of-All) now in its forty-second passage served as source material for the experimental cultures used in the present investigation.

The stock cultures were maintained in White's synthetic medium (White, 1943) containing 2 per cent. sucrose (S 2% S.). The medium, after autoclaving at 15 lb. for 15 minutes, has a pH of 4·6 to 4·8 (not 4·1 to 4·3 as previously reported in error). There is no evidence that the sucrose suffers any hydrolysis during the autoclaving. Standard culture medium (S 2% S.) prepared either (*a*) by autoclaving the sucrose solution separately and adding it aseptically to a sterile solution of the salts and vitamins, or (*b*) by dissolving solid sucrose aseptically in a sterile solution of the salts and vitamins, has been shown to support root growth qualitatively and quantitatively identical with that in the solution prepared by autoclaving. This contrasts with the work of Burström (1941 *b*), who states that in his excised root culture medium (Burström, 1941 *a*) sucrose is hydrolysed when autoclaved in the presence of the mineral salts.

Stock and experimental cultures were incubated during growth at 27° C. The stock cultures were subcultured every 7 days by White's technique.

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100-ml. wide-necked Hysil flasks containing 50 ml. of culture medium were used in the experiments, and growth was allowed to proceed for 7 days except where otherwise explicitly stated. The methods of expressing results and the calculation of significant differences have been described in a previous paper (Dormer and Street, 1949). The values for mean growth increments (mm.-culture-day) and for dry weights (mg.) are shown in the tables and in the text with their standard errors.

THE pH OF THE CULTURE SOLUTION

1. *The initial pH*

If the mechanism whereby excised tomato roots absorb sucrose involves the operation, at the cell surface, of an enzyme system it is to be expected that the pH of the culture medium will exert a marked effect upon root growth. White (1937), studying the effect of various inorganic nutrient solutions on the growth of excised tomato roots, noted that only those solutions which had a pH below 6·0 were satisfactory. Determining the pH of his own standard medium (50 ml. per culture) after it had supported growth of a root for 7 days, White found that medium of final pH 5·5 was superior to medium of pH 4·5 and markedly superior to media of pH 6·5 or higher. Hitherto no detailed study, with a standard inorganic solution, of the effect of initial pH on the growth of excised tomato roots has been published. Experiments were therefore undertaken to study the effect of the initial pH on the growth of our clone of excised tomato roots.

The pH of the standard culture medium (S 2% S.) was adjusted by the addition of N/20 NaOH or N/20 HCl. The pH adjustment did not cause any precipitation, and it was shown that sodium chloride when added to the standard medium in a concentration to give an excess of sodium or chloride ions over that involved in the pH adjustment was without significant effect on the growth rate. The pH of the medium, after autoclaving, was determined by the glass electrode and the mean value for four flasks, chosen at random, was taken as the pH value. The highest and lowest pH values within the four were required to differ by less than 0·3 pH unit for the batch of medium to be passed.

Experiment No. 1. This covered the pH range 3·65 to 6·75 and had 12 replicate cultures for each pH value.

Experiment No. 2. This covered the pH range 4·3 to 5·8 and had 20 replicate cultures for each pH value.

The results of these two experiments are shown in Fig. 1. These results indicate that good growth is only obtained within the pH range 4·2 to 5·0 with a fairly clearly defined optimum pH at 4·8 to 4·9. At pH values above 5·0 the growth rate falls off rapidly, laterals are inhibited, and the roots and particularly the root-tips become brown. On the acid side of the optimum pH growth is less seriously affected and down to pH 3·9 considerable growth and lateral formation take place. At the pH values 3·65 and 3·9 the roots are thin and translucent, resembling in this respect roots grown with dextrose as their carbon source (Dormer and Street, 1949). Whilst therefore initial pH values above 5·0 have a definitely toxic effect, this is not apparent at the acid end of the pH range tested where the symptoms resemble rather those which appear to be associated with carbohydrate deficiency.

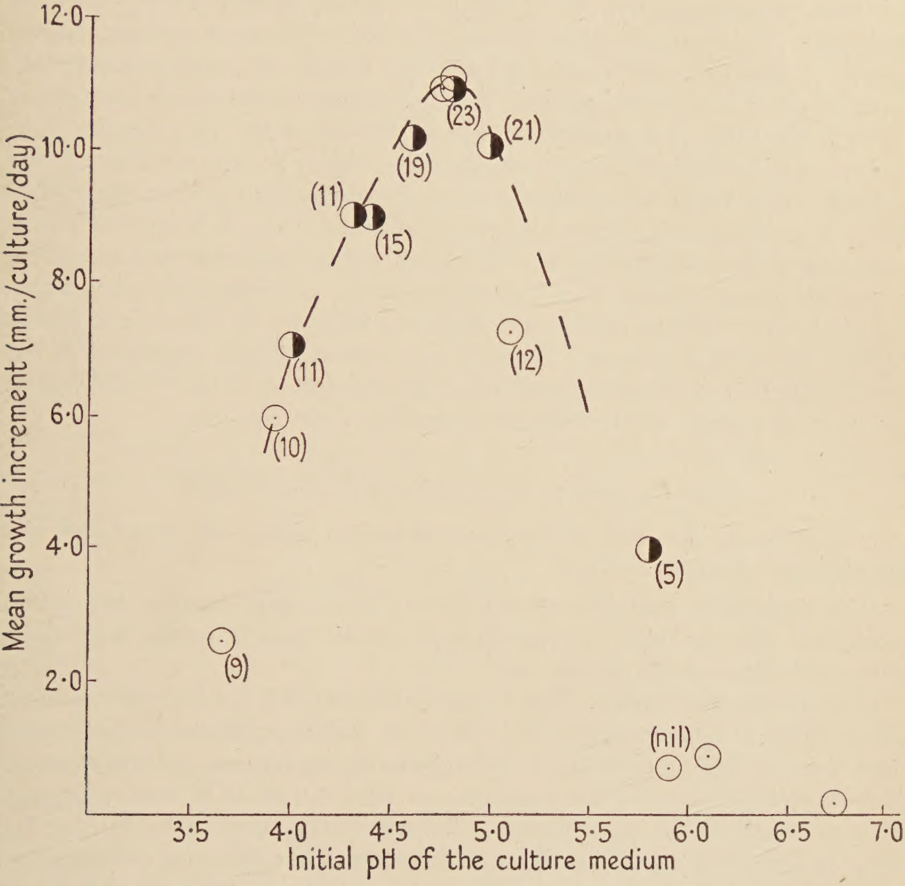

FIG. 1. The effect of initial pH of the culture medium on the growth of excised tomato roots. The mean number of laterals per root is indicated by the number against each mean growth increment value.

The marked pH sensitivity of excised tomato roots stands in contrast to the relative indifference to pH over a fairly wide range of the attached roots. Arnon and Johnson (1942), in a study of the effect of pH on the growth of tomato plants in water culture, reported almost equally good growth over the range pH 4 to pH 8. Excellent and uniform root growth occurred at pH values 5, 6, and 7 and only slightly retarded growth at pH values 4 and 8. Audus (1949), in a study of the growth of seedling cress roots in water culture, observed good growth over the range pH 5·0 to 8·5, with a uniform optimum between pH 5·0 and 6·5.

2. *The pH drift resulting from root growth*

Experiments have been conducted to follow the pH drift which occurs when roots are grown in the standard culture medium of initially favourable pH. Groups of three flasks were withdrawn at intervals during the duration of these experiments and the pH of the medium determined by the glass electrode. The mean pH values obtained in three such experiments are shown in Fig. 2. An alkaline drift in the pH occurs so that 50-ml. stock cultures reach a pH of 5·5 or over by the 7th day. The fall in growth rate which occurs after the 5th day (Fig. 3) is possibly related to this pH drift. This alkaline drift continues for at least 28 days, though the curve clearly flattens out after 14 days growth. Tips taken from cultures 14 or more days old and transferred to medium at pH 4·6 to 4·8 show a lag period of several days, before growth commences and the percentage of tips which fail to grow increases with increasing age of the parent culture. Tips from cultures 14 or more days old when transferred to new medium whose pH has been adjusted to that of the parent culture always fail to grow. There is no evidence of any adaptation of the developing root to growth in medium of alkaline pH, and the pH drift exerts an increasing check to the continuous growth of the cultures.

THE PHOSPHATE NUTRITION OF EXCISED ROOTS

1. *The effect of the level of inorganic phosphate supply on the growth and development of excised roots*

The hypothesis that phosphorylation is involved in sucrose absorption suggested that the level of inorganic phosphate supply might have quite a marked effect on the growth rate.

Experiments Nos. 3 and 4. The standard medium (S 2% S.) contains sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) at a concentration corresponding to *c.* 5 p.p.m. P(15 p.p.m. PO_4'''). Media containing various concentrations of this phosphate covering the range 0·0 to 60·0 p.p.m. PO_4''' were prepared. The pH of all media was adjusted to fall within the ranges pH 4·8 to 5·1 for Expt. 3 and pH 4·4 to 4·8 for Expt. 4. Root-tips from the stock cultures were grown for 7 days in the phosphate media, 20 cultures being used for each medium in both experiments. The results are shown in Fig. 4. Growth in length, lateral root formation, and root diameter were found to be indifferent to the phosphate concentration over the range 0·5 to 20·0 p.p.m. Roots growing in the minus-phosphate medium had, however, a significantly lower growth rate, laterals were either absent or one or two in number, and root diameter was reduced as compared with that of roots supplied with phosphate. The growth rate of these minus-phosphate roots declined during the experimental period and the growing apex became thin and delicate and showed a sharp change in direction. The contrast in dry weight between these roots and those grown with phosphate was more marked than the reduction in growth increment value: mean dry weight per root (mg.) for minus-phosphate $0·58 \pm 0·11$ against $2·15 \pm 0·20$ for 15·0 p.p.m. PO_4'''.

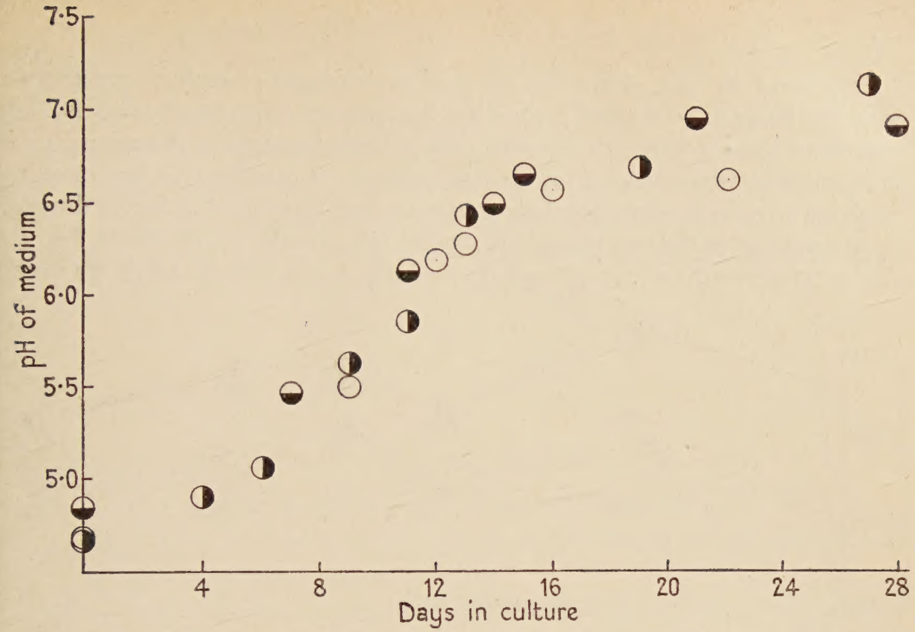

FIG. 2. The alkaline drift in pH occurring as a result of the growth of single root-tip inocula in 50 ml. of sucrose 2 per cent.-synthetic medium.

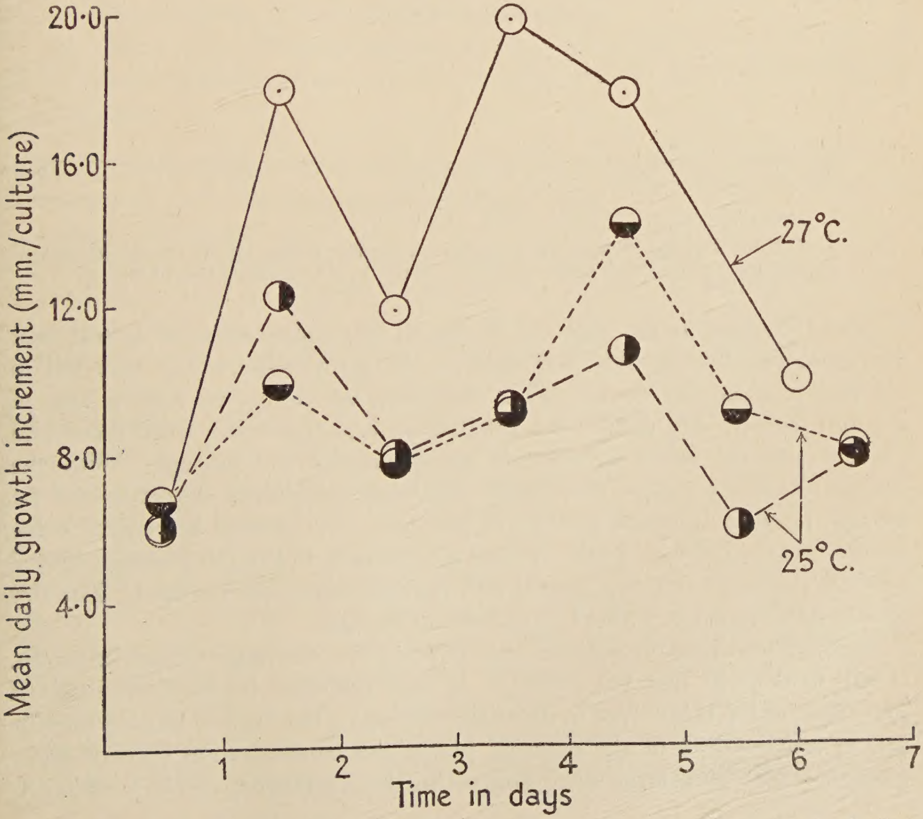

FIG. 3. Mean daily growth increment curves for excised tomato roots showing the decline in growth rate occurring after the fifth day in culture and the temporary check in growth frequently observed on the third day.

Experiment No. 4 a. Since the effect of sub-optimal phosphate concentrations was likely to be masked during the first passage from stock cultures, the roots from Expt. 4 were sub-cultured into phosphate media of the same phosphate concentrations as used for the first passage. A quantitative experiment, involving 20 cultures per phosphate concentration, was then conducted during the 5th passage in the test phosphate media. The results are shown in Fig. 4. The roots grown in minus-phosphate medium could only be taken through a second passage as tip cultures owing to the suppression of lateral root development. During this 2nd passage the growth increment rate fell to 1·9 mm./culture/day (mean of 14 cultures), as compared with a rate of 5·0 mm./culture/day during the 1st passage and tips taken from these 2nd passage cultures failed to grow in an attempted 3rd passage. With the concentration 0·5 p.p.m. phosphate deficiency symptoms absent in the 1st passage became apparent in the 5th passage. The growth increment value fell from 11·2±0·6 mm. in the 1st to 5·4±0·6 mm. in the 5th passage; lateral root development was suppressed and root diameter was reduced. The dry weight data shown in Table I emphasizes this also.

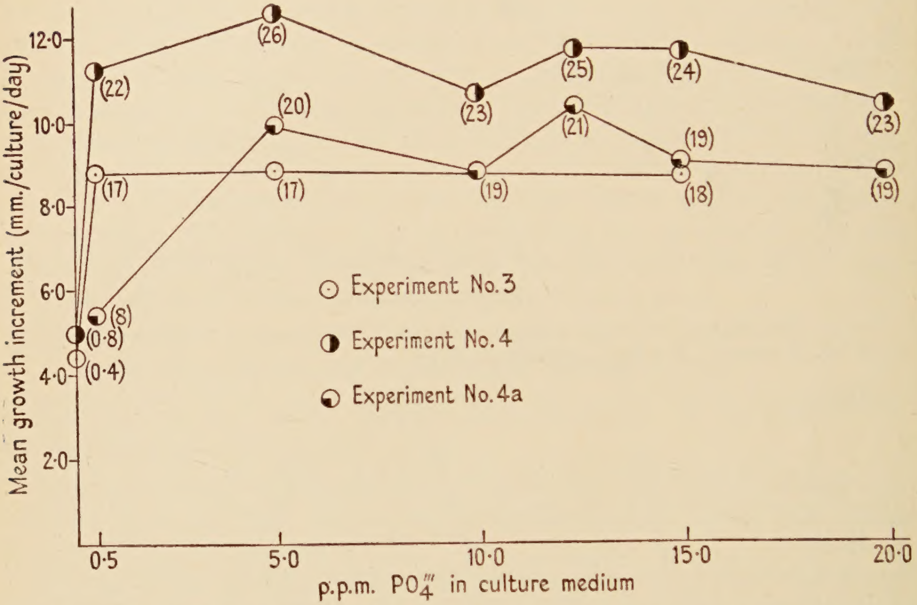

FIG. 4. The effect of the level of the inorganic phosphate supply on the growth of excised tomato roots. The mean number of laterals per root is indicated as in Fig. 1.

Indifference to the phosphate concentration over the range 5·0 to 20·0 p.p.m. is still evident in this 5th passage. It may therefore be concluded that, if phosphorylation is involved in sugar absorption by the excised tomato root, its rate is independent of the external phosphate concentration (except when phosphate starvation is induced), suggesting that a phosphate cycle, not directly influenced by the external phosphate concentration, must be operative within the cell.

TABLE I

(*Experiment 4a*)

p.p.m. PO_4'''	Mean dry wt. per root (mg.).
0·5	0·75±0·05
5·0	1·95±0·23
10·0	1·55±0·12
12·5	1·80±0·14
15·0	1·50±0·13
20·0	1·50±0·24

2. *The respiratory behaviour of phosphate-deficient excised roots*

Saïd (1948) reports, without giving actual experimental data:

(*a*) that the cut ends of leaves dipped in sucrose solution hydrolyse the sugar before absorbing it;

(*b*) that the increase in respiration rate resulting from sucrose feeding is reduced when the leaves are phosphorus-deficient and that this is associated with a reduced rate of inversion of the sucrose and a slower rate of uptake of the hydrolysis products;

(*c*) that feeding with neutral phosphate and sucrose together restores the normal hydrolytic and absorptive capacities of the phosphorus-deficient leaves.

On the basis of these observations, Saïd has advanced the hypothesis that phosphorus deficiency in leaves leads to reduced 'invertase' activity at the cell surfaces. An experiment was therefore undertaken to see if a similar behaviour characterized phosphorus-deficient excised tomato roots.

Experiment No. 5. Oxygen uptake of root sectors was determined by the direct method of Warburg. The Warburg flasks used were fitted with a centre well and single side arm. The centre well was charged with 0·2 ml. 10 per cent. w/v sodium hydroxide by the standard method. The body of the flask contained 2 ml. of S 0% S. minus phosphate solution to suspend the root sectors. The side arm contained 0·5-ml. treatment solution. 12 to 16-mm. root sectors were cut from the terminal 35 mm. of roots grown respectively in the standard S 2% S. (15 p.p.m. phosphate) medium and S 2% S. minus phosphate medium. The sectors were washed by transference to two changes of sterile S 0% S. minus phosphate solution and after 1 hour were transferred to the Warburg flasks. Each flask contained either 8×S 2% S. (15 p.p.m. phosphate) sectors or 12×S 2% S. minus phosphate sectors. The flasks were then fitted to their manometers and transferred to the bath at 27° C. and allowed to equilibrate for 15 minutes. The initial O_2 uptake per hour was determined over the period 0 to 2 hrs. The treatment solution was then tipped in from the side arm and the average O_2 uptake per hour determined over the periods 2 to 6 hrs., 6 to 18 hrs., and 18 to 22 hrs. The rates of O_2 uptake after addition of the treatment solutions are expressed as percentages of the initial rates in Table II. Each value in this table is the mean of six separate runs, performed with separate batches of root material. There were no signs of bacterial contaminations in any of the flasks at the end of the runs and the thermobarometer, which always contained S 2% S.+15 p.p.m. phosphate, never showed any significant uptake of oxygen. The S 0% S. minus phosphate solution used was sterile and the treatment solutions were freshly prepared with fresh double-distilled water and were stored in the refrigerator. Sectors from roots grown on S 2% S. (15 p.p.m. phosphate) medium when suspended in S 0% S. (15 p.p.m. phosphate) solution showed a fall in the rate of O_2 uptake during the period 2 to 6 hours to give a rate, subsequently maintained during the whole experiment and corresponding to 60 to 65 per cent. of the initial rate. When these sectors were supplied with sucrose either in the absence or presence of external phosphate a steady rise in the O_2 uptake took place until by the end of the experimental period the rate was 200–250 per cent. of the initial rate.

TABLE II

Experiment No. 5: Oxygen Uptake of Root Sectors obtained from Excised Tomato Roots (Suspending medium S 0% S. minus phosphate.)

Root sectors from	Experimental treatment (commencing at 2 hrs.)	Mean values for oxygen uptake per hour during treatment expressed as a percentage of oxygen uptake per hour during the period 0 to 2 hrs.		
		2 to 6 hrs.	6 to 18 hours	18 to 22 hrs.
Normal roots (grown on S 2% S.+15 p.p.m. PO_4''')	15 p.p.m. PO_4'''	65	64	60
	Sucrose 2%	112	141	201
	15 p.p.m. PO_4'''+ +Sucrose 2%	83	117	256
Phosphorus-deficient roots (grown on S 2% S. minus PO_4''')	15 p.p.m. PO_4'''	105	99·5	123
	Sucrose 2%	104	95	86
	15 p.p.m. PO_4'''+ Sucrose 2%	127	188	457

Sectors from roots grown on S 2% S. (minus phosphate) medium had a low rate of respiration which was, however, well maintained when the sectors were suspended in S 0% S. (15 p.p.m. phosphate) solution. When supplied with sucrose in the absence of external phosphate a slow decrease in the O_2 uptake of the sectors took place, but when supplied with sucrose and phosphate simultaneously a rapid increase in the rate of O_2 uptake occurred so that by the end of the experimental period the rate achieved was some 450 per cent. of the initial rate. The phosphorus-deficient root sectors closely paralleled in respiratory behaviour the phosphorus-deficient leaves used by Saïd.

The rapid restoration of the sucrose absorbing ability of both phosphorus-deficient leaves and roots resulting from an external supply of inorganic phosphate strongly suggests a phosphate activation of the absorbing mechanism rather than a specific role for phosphate in the synthesis of the enzyme(s) involved in sugar utilization. The absence of any direct evidence for an activation of 'invertase' by phosphate therefore also from the invertase hypothesis and enhances the view now postulated that a phosphorolysis of sucrose is involved in sugar absorption.

THE PHLORIDZIN INHIBITION OF ROOT GROWTH

Lundsgaard (1933) and Kalckar (1937) suggested that phloridzin prevents glucose reabsorption in the proximal tubules of the kidney by inhibiting the phosphorylation of glucose. Ostern *et al.* (1936) showed that phloridzin prevents the formation of glucose-6-phosphate in muscle extracts incubated with glycogen and inorganic phosphate. Dahl (1939) obtained similar results. Cori, Colowick, and Cori (1939) and Cori and Cori (1940) studying the kinetics of glycogen synthesis from glucose-1-phosphate reported phloridzin and phloretin to be inhibitors of glucosan phosphorylase. Phloridzin at 0·01 M caused 85 per cent. or greater inhibition of phosphorylase activity. Shapiro (1947), using minced rat kidney tissue, found certain dehydrogenases to be much more sensitive to phloridzin poisoning than the glucose phosphorylating system, though the latter was reported to be significantly inhibited by 0·001 to 0·003 M phloridzin.

Lisitsyn (1941) is reported by Hartt (1943) to have found that 0·005 M phloridzin inhibited the synthesis of sucrose in the leaves of *Arctium lappa* and *Crataegus* sp. but not in the leaves of *Tussilago farfara*. Kriukova (1940), however, failed to detect inhibition of sucrose synthesis in leaves infiltered with phloridzin, and Hartt (1943), without giving experimental details, reported that phloridzin at 0·01 M concentration did not interfere with sucrose synthesis in detached leaf blades of sugar-cane. Little significance can, however, be attached to these negative results as there is no evidence that, with the experimental conditions employed, the phloridzin was brought into contact with the sucrose synthesizing system. Doudoroff (1943) has, however, shown that phloridzin, at concentrations between M/400 and M/150, is without significant effect on the activity of bacterial sucrose phosphorylase. Whilst therefore phloridzin inhibits certain phosphorylations, there is no reliable data concerning its effect on sucrose phosphorolysis or synthesis in higher plants. It was, however, considered desirable to study the action of phloridzin on the utilization of sucrose by excised tomato roots in view of its general ability to inhibit phosphorylating systems.

1. *Purification of phloridzin*

The commercial phloridzin available was a very pale yellow crystalline powder with a solubility of about 1 g. in 1,000 g. water at 20° C. The saturated aqueous solution was almost colourless when prepared but gradually turned bright yellow on storage, particularly when kept on the alkaline side of neutrality or exposed to light. The standard culture medium containing 0·1 per cent. w/v of this commercial phloridzin turned bright yellow on autoclaving at 10 lb. for 15 minutes. No precipitation of phloretin (insoluble in water) or significant change in pH occurred, and it was therefore concluded that the coloration was not associated with hydrolysis of the glucoside but was probably due to the presence of impurity. When commercial phloridzin was purified by the procedure described below, which is a modification of that used by Moelwyn-Hughes (1928), it was obtained as colourless needles. These dissolved in water to give a colourless solution which developed little or no coloration on autoclaving and only turned pale yellow after 7 days' incubation at 27° C.

Procedure. 15 g. of commercial phloridzin were dissolved in 1 litre of distilled water at 60° C. and the solution set aside in the refrigerator at 4° C. overnight. The crystals were collected and redissolved in 1 litre of distilled water. The solution was heated in a water-bath to 75° C. to 80° C. and treated for 10 minutes with 10 g. of activated charcoal. The solution was filtered and set aside to crystallize overnight in the refrigerator. The crystals were collected and recrystallized a third time from 1 litre of distilled water. The crystals were collected on a No. 50 Whatman filter and dried in a desiccator over phosphorus pentoxide. This purified phloridzin was used in the experiments described below.

2. *Effect of phloridzin concentration on the growth of excised root supplied with 2 per cent. sucrose*

Experiment No. 6. Phloridzin was added to the standard culture medium (S 2% S.) to give the following concentrations: 0·1 (0·0021 M), 0·05, and 0·01 per cent. The media were sterilized by autoclaving at 10 lb. for 15 minutes. The experiment involved 40 cultures; 10 replicates of each medium being inoculated.

Experiment No. 7. S 2% S. medium containing the following concentrations of phloridzin was employed: 0·1, 0·07, 0·05, 0·03, and 0·01 per cent. The experiment involved 120 cultures; 20 replicates of each medium being inoculated.

The results of these experiments are shown in Table III. Phloridzin inhibited growth in length and lateral root formation and at the higher concentrations caused a reduction in root diameter. The inhibitory effect on both growth in length and lateral formation increased regularly with increasing concentration of phloridzin up to the highest concentration used (0·1%). The absence of any obvious toxic symptoms supported the view that phloridzin interferes with the carbohydrate nutrition of the excised root. The inhibitory effect of phloridzin on growth in length is shown by calculation of the 'percentage inhibition' from the equation:

$$\text{Percentage inhibition} = \frac{(\text{S } 2\%\text{ S.}) - (\text{S } 2\%\text{ S. Phloridzin})}{(\text{S } 2\%\text{ S.})} \times 100,$$

where (S 2% S.) and (S 2% S. phloridzin) are the mean growth increments in mm.-culture-day respectively of the standard medium (control cultures) and the standard medium containing the test concentration of phloridzin.

TABLE III

The Effect of Phloridzin on Excised Roots growing in Synthetic Medium containing 2 per cent. Sucrose

Experiment No.	Character measured	Percentage phloridzin.					
		0·0 control.	0·01	0·03	0·05	0·07	0·10
6	Mean percentage inhibition of growth in length . .	—	22·3	—	48·6	—	73·4
7	Mean percentage inhibition of growth in length . .	—	26·0	42·0	55·8	58·6	67·3
	Mean number of laterals per root .	23	18	10	6	5	1

3. *Effect of the concentration of sucrose on the inhibition of growth caused by phloridzin*

Experiment No. 8. The standard culture medium containing 1, 2, and 3 per cent. sucrose was used. Half the cultures contained 0·04 per cent. phloridzin. The experiment involved 120 cultures; 20 replicates of each medium being inoculated. The results are shown in Table IV. It is clear that the degree of inhibition decreases with increasing concentration of sucrose, strongly suggesting competition between sucrose and phloridzin for an enzyme system essential for the carbohydrate nutrition of the excised root.

TABLE IV

The Effect of the Concentration of Sucrose on the Inhibition of Excised Root Growth caused by 0·04 per cent. Phloridzin (Data from Experiment No. 8)

Character measured.	Percentage sucrose.		
	1·0	2·0	3·0
Mean percentage inhibition of growth in length caused by 0·04 per cent. phloridzin . .	64·3	43·0	25·1
Mean number of laterals per root in the controls	12	19	10
Mean number of laterals per root in presence of 0·04 per cent. phloridzin	0	10	10

4. *The reversibility of phloridzin inhibition*

Experiment No. 9. Two groups (*A* and *A′*) of twenty 4-day-old cultures in sucrose 2 per cent. synthetic medium and two similar groups (*B* and *B′*) in sucrose 2 per cent. synthetic–phloridzin 0·05 per cent. medium were measured and the mean growth increment of each group calculated. The roots from the first group (*A*) in S 2% S. medium were then transferred aseptically to S 2% S. medium previously brought to 27° C. in the incubator. The roots from the second group (*A′*) were then transferred aseptically to new S 2% S. phloridzin 0·05 per cent. medium also at 27° C. The roots from the first group (*B*) in S 2% S. phloridzin 0·05 per cent. medium were transferred to new S 2% S. phloridzin 0·05 per cent. medium, and the roots from the second group (*B'*) were transferred to new S 2% S. medium by the same technique. All cultures were measured and the laterals counted at the end of the 3rd day following transference (168 hours from the initiation of the cultures). The results are summarized in Table V. It is clear that an initial period of 4 days in the standard medium mitigates the effect of phloridzin and eliminates the decrease in lateral root formation normally associated with phloridzin inhibition. A 4-day period of moderate phloridzin inhibition does not prevent a subsequent increase in growth rate when the roots are transferred to phloridzin free medium.

TABLE V

Reversibility of the Phloridzin Inhibition of Excised Root Growth (Data from Experiment No. 9)

0 to 96 hours		96 to 168 hours		
Culture medium.	Mean value mm./culture/day during period.	Mean value mm./culture/day during period.	Mean number of laterals per root at 168 hours.	Culture medium.
Sucrose 2%-synthetic (*A*)	6·1±0·7	11·1±1·9	15	Sucrose 2%-synthetic
Sucrose 2%-synthetic (*A'*)	6·8±0·6	6·6±0·6	15	Sucrose 2%-synthetic+ 0·05% phloridzin
Sucrose 2%-synthetic+ 0·05% phloridzin (*B*)	3·5±0·3	3·5±0·3	3	Sucrose 2%-synthetic+ 0·05% phloridzin
Sucrose 2%-synthetic+ 0·05% phloridzin (*B'*)	3·6±0·3	5·2±0·4	5·5	Sucrose 2%-synthetic

Experiment No. 10. The roots of three groups of twenty-two 2-day-old cultures in S 2% S. medium were measured. Using the technique described in Expt. 9, the roots of one group were transferred to new S 2% S. medium, and the roots of the other two groups transferred to S 2% S. phloridzin 0·1 per cent. medium. After 48 hours the roots were measured and the mean growth increment for each group determined. The roots in S 2% S. medium were then transferred to new S 2% S. medium and one group of the roots in S 2% S. phloridzin 0·1 per cent. medium transferred to new S 2% S. phloridzin 0·1 per cent. medium. The second group of roots in S 2% S. phloridzin 0·1 per cent. medium were transferred to new S 2% S. medium. After 48 hours the roots were again measured and the laterals counted. The results are summarized in Table VI. The 2-day period in S 2% S. medium protects the roots from the full inhibitory effect of phloridzin, and roots inhibited by a concentration of phloridzin as high as 0·1 per cent. quickly show an increase in growth rate when transferred to phloridzin-free medium. The 2-day period in S 2% S. medium is also sufficient to reduce the inhibitory effect of phloridzin on lateral root formation and the development of laterals increases significantly if the 2-day phloridzin treatment is followed by removal to phloridzin free medium.

Mean growth increments for each separate 24 hours, during the 7-day growth period in S 2% S. medium, are shown in Fig. 3. The data from

TABLE VI

Reversibility of the Phloridzin Inhibition of Excised Root Growth (Data from Experiment No. 10)

48 to 96 hours		96 to 144 hours		
Culture medium.	Mean value mm./culture/day during period.	Mean value mm./culture/day during period.	Mean number of laterals per root at 144 hours.	Culture medium.
Sucrose 2%-synthetic	13·1±1·1	18·0±1·4	19	Sucrose 2%-synthetic
Sucrose 2%-synthetic + 0·10% phloridzin	4·9±0·4	5·6±0·4	6	Sucrose 2%-synthetic + 0·1% phloridzin
Sucrose 2%-synthetic + 0·10% phloridzin	4·5±0·3	11·2±0·8	11	Sucrose 2%-synthetic

Expts. 9 and 10 suggests that lateral initiation takes place early in the growth period and that many of the laterals visible after 7 days are formed during the first 96 hours. The significant decrease in growth increment rate frequently noted on the third day of growth in S 2% S. medium (Fig. 3) is therefore probably related to the diversion of food material to lateral root meristems. This early lateral root formation would explain why phloridzin treatment subsequent to 48 hours fails to suppress completely the development of laterals and if applied after 96 hours fails even to reduce their number (Expt. 9).

5. *Effect of phloridzin on the growth of seedling roots*

The reversibility of phloridzin inhibition, its proportionality to phloridzin concentration, and the ability of increased external sucrose concentration to reduce significantly the effect of the phloridzin strongly suggest that this substance acts as a competitive inhibitor of sucrose absorption. The possibility remained, however, that phloridzin penetrates into the cells in significant amounts, inhibits growth by interference with the general cell metabolism, and that the reversibility of phloridzin inhibition is due to operation of a detoxicating mechanism. Such an hypothesis, postulating a general effect on cell metabolism rather than a specific surface effect on the enzyme system mediating sucrose absorption, would be supported if the seedling root receiving as it does an internal sugar supply from the seed should show a similar phloridzin inhibition to the excised root.

Experiment No. 11. Sterile seedling cultures were set up by the technique previously described (Dormer and Street, 1949), using as media S 0% S. and S 0% S.+0·07% phloridzin. The seedlings (35 per treatment) were harvested 8 days after surface sterilization of the seed and the length of each radicle measured. The results obtained for the mean radicle lengths (in mm.) were: Control S 0% S. = 39·0±1·61; 0·07% phloridzin = 42·0±1·85. No significant difference in length was found. In general appearance the seedlings grown in the control and in the phloridzin medium were indistinguishable. The rather large standard error recorded for these seedling roots is due to the variable time of germination within each treatment. Excised roots growing in sucrose medium when treated with the same concentration of phloridzin suffer a 58 per cent. inhibition of their growth in length. The absence of any inhibition of the growth of the seedling roots is considered to support strongly the view that phloridzin inhibition of the excised roots results from a retardation of sucrose absorption rather than from any toxic action of the glucoside on general cell metabolism.

THE EFFECT OF VARIOUS SUGARS ON THE GROWTH OF EXCISED ROOTS SUPPLIED WITH SUCROSE

Doudoroff (1943) has shown that dextrose inhibits the action of the sucrose phosphorylase of *Pseudomonas saccharophila*. Later Doudoroff, Barker, and Hassid (1947) found that xylose, arabinose, and galactose have a much weaker inhibitory action on this bacterial sucrose phosphorylase than does dextrose.

1. *The inability of various sugars to support the growth of excised roots*

The habit and growth rate of excised tomato roots supplied with dextrose as the sole carbon source has been described in a previous paper (Dormer and Street, 1949). A number of other sugars have been tested in our laboratory for their ability to support the growth of excised tomato roots. Galactose (2 samples), mannose (2 samples), arabinose, xylose, rhamnose, and maltose (2 samples), all used at 2 per cent. concentration in the standard synthetic medium, proved unable to support growth: the standard 10-mm. root-tip inocula when supplied with these sugars shows a total extension of less than 5 mm. in 7 days. Two samples of laevulose failed to support growth, whilst a third sample supported a mean daily extension of only 1·4 mm. for 5 days, after which growth ceased. This extension was associated with a decrease in root diameter and a curling of the tip (a feature frequently noted in dextrose grown roots). Raffinose (2 samples), used at 3 per cent. concentration, supported a rate of extension ranging from 0·7 to 1·0 mm. per day, growth frequently ceasing before completion of the 7-day incubation period. Laevulose and raffinose are clearly unable to support even the slow but maintained rate of growth obtained with dextrose media. The other sugars tested as alternatives to sucrose have proved therefore, with the exception of dextrose, unable to support the growth of excised tomato roots. The experiments described below were undertaken to study the effect of these sugars on the growth of excised tomato roots simultaneously supplied with sucrose.

2. *The inhibitory effect of various sugars on the growth of excised roots supplied with sucrose*

Experiment No. 12. This experiment involved 7 culture media and 20 replicate cultures for each medium. All completed media were sterilized by autoclaving at 10 lb. for 15 minutes. A preliminary experiment had shown that growth in raffinose-containing media was not significantly affected by autoclaving the sugar and salts separately as against autoclaving the complete media. The root-tips were grown in the experimental media for 5 days. The results are shown in Table VII. Galactose, maltose, xylose, and laevulose, at the concentration used, almost completely inhibited the growth of roots simultaneously supplied with 1 per cent. sucrose. White (1940) has found galactose, maltose, and laevulose unable to support the growth of excised tomato roots and described definite toxic symptoms developed in their presence. Burström (1948) has reported that galactose is not only unable to support the growth of excised wheat roots but has a specific toxic action. Wheat roots readily utilized dextrose as a carbon source, but the addition of galactose to the dextrose medium not only inhibited root growth but caused rapid death of the meristem. The inability of excised tomato roots to grow in presence of galactose, maltose, xylose, and laevulose probably results from a similar toxic action rather than from a marked inhibition of the sucrose absorbing mechanism by these sugars. The accumulation of laevulose during the growth of excised tomato roots supplied with sucrose (Dormer and Street, 1949) would appear to be an important 'staling' factor. Raffinose exerted no deleterious effect upon growth and it was noted that its presence was associated with a more even growth of the replicate cultures than occurred in the S 1% S. medium.

TABLE VII

The Effect of Various Sugars on the Growth of Excised Tomato Roots simultaneously supplied with 1 per cent. Sucrose. Growth Period 5 Days. (Data from Experiment No. 12)

Culture medium.	Initial pH.	Mean growth increment mm./culture/day.	Mean number of laterals per root.
S 1% S.	4·6	10·1±1·3	12
S 1% S.+1% Dextrose	4·3	5·3±0·6	9
S 1% S.+1% Laevulose	4·3	1·3±0·2	1
S 1% S.+1% Galactose	4·3	0·30±0·04	—
S 1% S.+1% Xylose	4·5	1·5 ±0·3	0·2
S 1% S.+2% Maltose	4·2	0·9 ±0·2	1
S 1% S.+3% Raffinose	4·7	11·9 ±0·7	15

3. *Dextrose as an inhibitor of sucrose utilization*

Experiment No. 13. This experiment was designed to study the effect of sucrose concentration on root growth and to see how far the inhibitory effect of 1 per cent. dextrose would be dependent upon the concentration of sucrose simultaneously supplied. It involved 10 culture media and 20 replicate cultures for each medium. The media were adjusted in pH so that, after autoclaving, the extreme limits of initial pH for the experiment were 4·5 to 5·0. The small pH differences between the experimental media did not obscure the effects on root growth of sucrose concentration and of the presence of dextrose. The results are summarized in Fig. 5.

Optimum growth was obtained with 2 per cent. sucrose. With 0·1 per cent. and 0·5 per cent. sucrose the roots resembled those grown in dextrose media showing the reduction in root diameter and absence of laterals. The deleterious effect of high sucrose concentration, previously observed with 4 per cent. sucrose (Dormer and Street, 1949), is noteworthy. At 5 per cent. concentration a very high percentage of the roots are 'sinkers' (White, 1943, p. 140). This enhanced specific gravity, as it is associated with thickening of the root axis, suggests carbohydrate accumulation rather than an inhibition of air-space development.

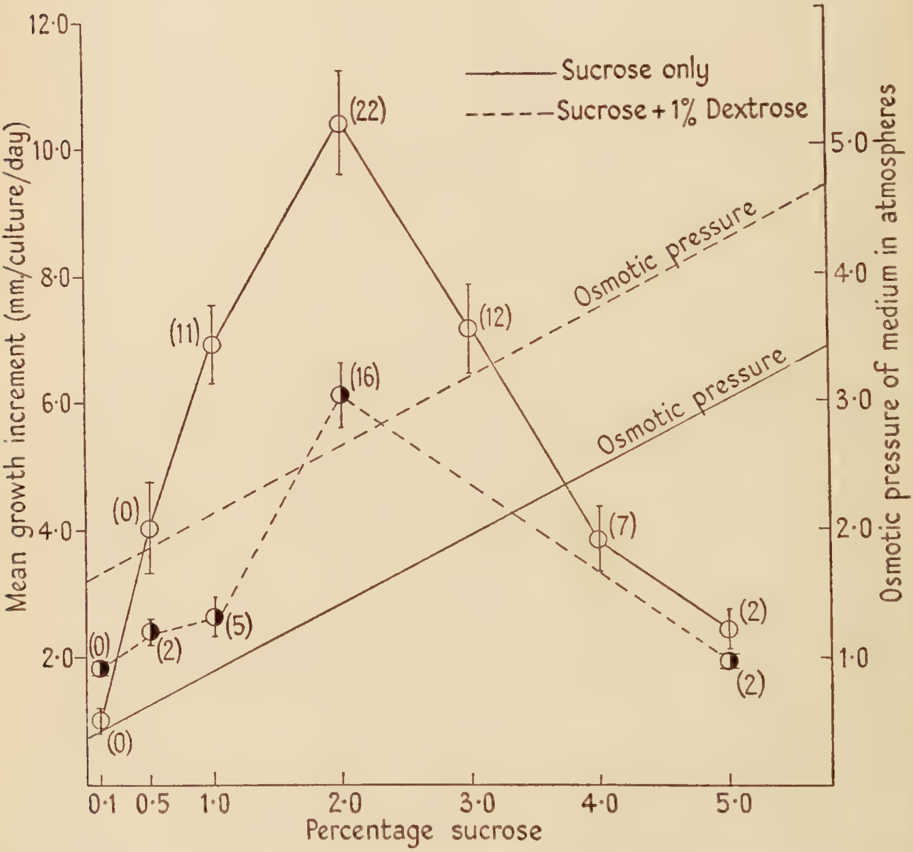

FIG. 5. The effects on the growth of excised tomato roots of the sucrose concentration and of the addition of dextrose to sucrose-containing media. The lengths of the vertical black lines represent twice the standard errors of the mean growth increment values plotted with the mean as a mid-point. The mean number of laterals per root is indicated as in Fig. 1.

The addition of 1 per cent. dextrose to solutions containing 0·5, 1·0, and 2·0 per cent. sucrose caused a decrease in root growth and with 1·0 and 2·0 per cent. sucrose a decrease in the mean number of laterals per root. The proportional decrease in growth rate caused by 1 per cent. dextrose in presence of 1 per cent. sucrose was more marked than in presence of 2 per cent. sucrose, suggesting that dextrose causes a competitive inhibition of sucrose utilization. The presence of dextrose was not associated with toxic symptoms and with 0·1 per cent. sucrose the addition of 1 per cent. dextrose actually raised the growth rate slightly, the roots being identical in appearance with dextrose-grown roots, suggesting that dextrose had displaced sucrose as the main carbon source.

4. *The effect of sucrose concentration and osmotic pressure on root growth*

Experiment No. 14. This experiment was designed to study the effect of 3 per cent. raffinose on the growth of roots supplied with various concentrations of sucrose and the effect of sucrose concentration on root growth when all solutions are adjusted to the same osmotic pressure by raffinose. It involved 11 culture media and 19 replicate cultures for each medium. After autoclaving the initial pH values of the media fell within the limits pH 4·55 to 4·85, with the exception of S 0·5% S.+3 per cent. raffinose medium which had an initial pH of 5·1. The results are summarized in Fig. 6.

This experiment confirmed that with the standard synthetic medium 2 per cent. sucrose is superior to the other sucrose concentrations tested and that raffinose does not cause any retardation of the growth of excised tomato roots simultaneously supplied with sucrose.

In both Expts. 13 and 14 it was observed that the roots grown with 1·0 per cent. or less sucrose were delicate in structure and that with 0·5 per cent. or less sucrose they are clearly limited in growth by deficiency of carbohydrate. Roots receiving 3 per cent. or more sucrose were characterized by an increase in diameter and yellowing of the main axis as compared with roots grown in the standard medium (S 2% S.), and by a reduction in the length of the laterals. At 4 per cent. and 5 per cent. sucrose concentration the laterals were reduced in number and were distorted. At 4 per cent. sucrose some 40 per cent. and at 5 per cent. some 80 per cent. of the roots examined were 'sinkers'.

By using raffinose (Expt. 14) to adjust the osmotic pressure of media containing 1, 2, and 3 per cent. sucrose so that they were equal in O.P. to S 4% S. medium it became evident that the deleterious effect of 4 per cent. sucrose is not due to the enhanced O.P. of the medium but to the concentration of sucrose itself. This combined with the data presented above suggests that high sucrose concentrations are deleterious to root growth because they derange the normal carbohydrate metabolism of the roots.

The addition of raffinose to S 1% S. medium enhanced the growth rate and uniformity of development assessed after 7 days' incubation. This effect, presumably due to increase in the osmotic pressure of the medium, is noticeable even after only 5 days' incubation (Expt. 12). The inhibition of growth caused by the addition of 1 per cent. dextrose to S 1% S. medium is therefore very marked compared with that occurring in presence of 2 per cent. sucrose for the dextrose in enhancing the O.P. of the S 1% S. medium should be beneficial to root growth. The inability of raffinose to enhance the growth of roots supplied 0·5 per cent. sucrose confirmed that raffinose is unable to act as a carbon source for the growth of excised tomato roots and that at this sucrose concentration the extent of root growth is controlled by a deficiency of utilizable carbohydrate.

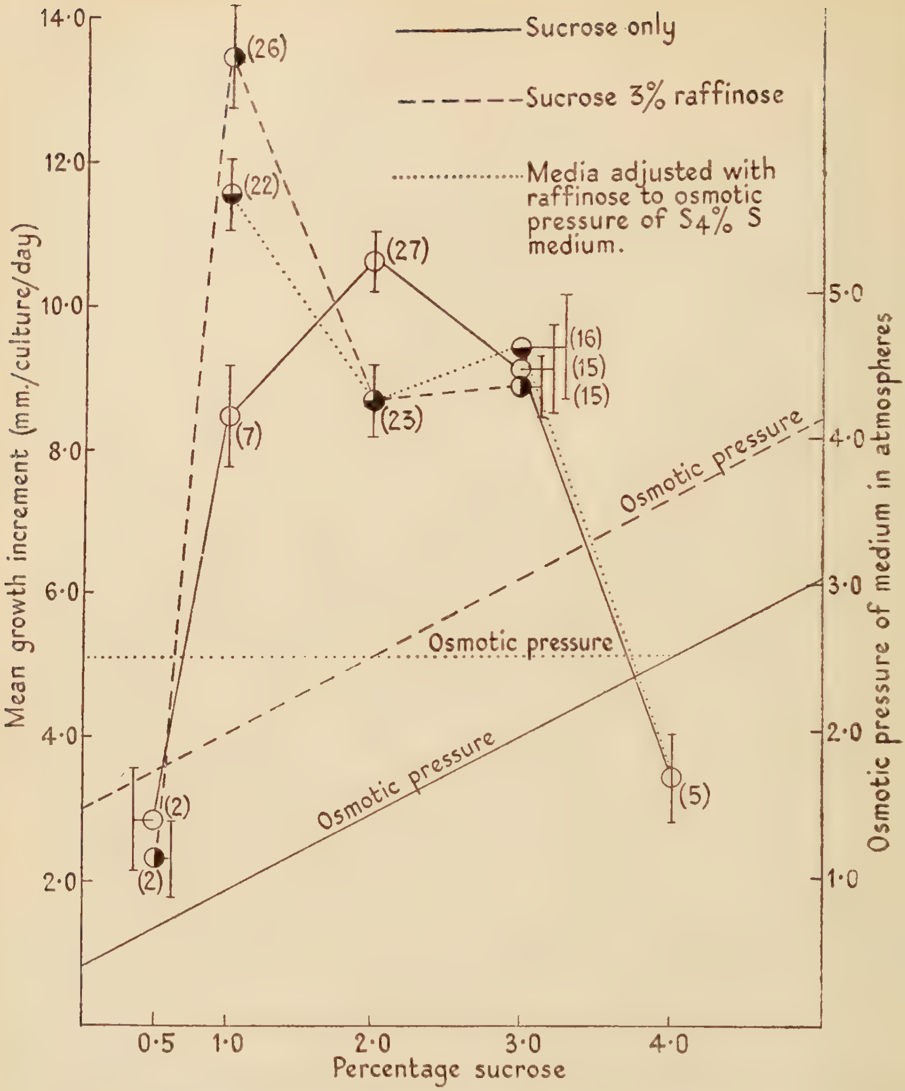

FIG. 6. The effects on the growth of excised tomato roots of the sucrose concentration and of the addition of raffinose to sucrose-containing media. Standard errors are indicated as in Fig. 5 and the mean number of laterals per root as in Fig. 1.

DISCUSSION

Recent studies of cell permeability have supported the hypothesis that there exists at the protoplast surface a bi-molecular layer of lipoidal units which is overlaid with unfolded protein units orientated tangentially with the surface (Davson and Danielli, 1943). This surface layer represents the main energy barrier to the entry of solutes into the cytoplasm and leads to the conception that entry involves penetration of the lipoid-protein film, passage through the film, and liberation into the cytoplasm. Such a film as that postulated will offer a marked resistance to the intake of highly polar molecules such as sugars.

The high content of protein in the surface film and its undoubted metabolic activity, as evidenced by cell-wall formation, makes it possible that a number of enzymes and particularly those involved in carbohydrate synthesis and degradation may be located in the protoplast surface. A number of workers have obtained evidence of such a surface localization of enzymes. Wilkes and Palmer (1933) and Nelson and Wilkes (1933) have shown that the sucrose 'inverting' enzyme of yeast cells immediately responds in activity to external pH and sucrose concentration as if it were in cell-free solution, thereby strongly suggesting its localization in the cell surface and in direct contact with the external solution. Myrback and Vasseur (1943) have similarly presented evidence for the localization of a lactase on the surface of various yeasts. The surface localization of various phosphatases in animal cells and yeasts has been demonstrated by cytochemical techniques (Dempsey and Deane, 1946; Emmell, 1946) and by the use of isotopic phosphorus (Rothstein and Meier, 1948). Sacks and Altshuler (1942) and Sacks (1944, 1948) have obtained evidence that phosphate absorption by muscle, heart, and liver involves the formation and later degradation on the cell surface of a hexosemonophosphate. This data, together with that cited in our previous paper (Dormer and Street, 1949), all supports the view that certain enzymes, and particularly those concerned with the phosphorylation and dephosphorylation of sugars, are present in the protoplast surface.

The absorption of sucrose by storage tissues (Gawadi, 1935; Saïd, 1941), leaves (Saïd, 1948) and excised roots (Burström, 1941 *b*; Dormer and Street, 1949) is associated with the appearance of a mixture of dextrose and laevulose in the external solution. The laevulose accumulates in a quantity corresponding closely to the theoretical yield to be expected from the sucrose degraded. Sugar absorption involves almost exclusively the dextrose moiety of the sucrose molecule.

Burström (1941 *b*), in his study of the carbohydrate nutrition of excised wheat roots, has advanced the hypothesis, based on Lundegårdh's (1940) studies of the properties of the plasm surface, that sucrose suffers an acid hydrolysis at the acid pH of the root surface, and that sucrose hydrolysis is not directly related to its absorption but is merely a function of the root surface area. Sucrose is, however, toxic to wheat roots and in their presence suffers a very rapid hydrolysis during which it is postulated injurious products arise from the fructose unit. Burström, from a preliminary study of sucrose utilization by Petkuser-rye roots, has suggested that a similar acid hydrolysis occurs, although at only one-tenth the rate observed with wheat, presumably due to the less acid pH at the root surface. The data in this case does not, however, exclude the possibility that a breakdown related to absorption is proceeding simultaneously with an acid hydrolysis. Rye roots apparently grow well when supplied either with dextrose or sucrose. Growth with sucrose is not associated with any toxic symptoms and is more rapid than with an equimolecular dextrose solution. This Burström explains as due to the higher amount of disposable nutrients, although no comparison of growth with equal percentages of the two sugars is recorded.

Gawadi (1935) put forward the view that the appearance of fructose and dextrose in the external solution observed when discs of carrot root tissue were supplied with sucrose was due to the activity of invertase centres on the outer surface of the cytoplasm. Saïd (1941), using discs of carrot and radish root tissue, accepted this hypothesis and in support cited the enhanced sucrose inversion occurring when the thickness of the tissue discs was decreased. These workers visualize invertase hydrolysis to precede utilization of sucrose so that entry of sugar takes place mainly as dextrose and to a smaller extent as fructose. On the basis of this hypothesis Saïd (1948), from his observations on sucrose utilization by detached leaves, has postulated a phosphate activation of the invertase centres.

Whilst certain tissues may effect a hydrolysis of sucrose either by virtue of the acid pH or invertase activity of the plasm surface it is clear, from the following considerations, that a quite different mechanism is involved in sucrose utilization by the excised tomato root:

(*a*) Sucrose supports a growth outstandingly superior to that observed with any other sugar tested.

(*b*) Neither dextrose nor laevulose nor an equimolecular mixture of these two sugars are capable of supporting a growth in any way comparable to that obtained by using sucrose.

(*c*) Sucrose breakdown is quantitatively related to its utilization, one 'dextrose' unit being absorbed for each two molecules of sucrose degraded (Dormer and Street, 1949).

(*d*) The growth responses to changes in the pH of the medium are quite different from those required by the acid hydrolysis hypothesis (Burström, 1941 *b*) but suggest rather the existence of an enzyme controlled mechanism.

Hartt (1944) and more recently Calvin and Benson (1949) have obtained evidence that sucrose synthesis in the green plant takes place by a dephosphorylative condensation. Further, the sugar requirements of *Pseudomonas saccharophila* (Doudoroff, 1940), from which Doudoroff (1943) has isolated a sucrose phosphorylase, are almost identical with those of excised tomato roots. Other studies, previously reviewed (Dormer and Street, 1949), emphasize the possibility that active sugar absorption by animal cells involves phosphorylation at the cell surface followed by dephosphorylation, catalysed by surface-localized phosphatases, as the sugar is liberated into the cytoplasm. It was therefore suggested that the specificity of sucrose as a carbon source for the growth of excised tomato roots and the relationship between sucrose breakdown and sugar absorption would be explained if the mechanism of utilization involved a phosphorolysis of the sucrose at the surface of the absorbing cells. This hypothesis is now supported by the following additional evidence:

(*a*) The similarity between the symptoms of phosphorus- and carbohydrate-deficiency.
(*b*) The demonstration of a phosphate activation of sucrose absorption by phosphorus-deficient roots.
(*c*) The reversibility and proportionality to concentration of the inhibition of excised root growth resulting from phloridzin treatment and the demonstration that this inhibition can be partially counteracted by increasing the sucrose concentration. The view advanced that phloridzin acts as an inhibitor of a phosphorylation involved in sucrose absorption is strengthened by the insensitivity of the attached seedling root to phloridzin treatment.
(*d*) The evidence that dextrose is a competitive inhibitor of sucrose utilization.

Further evidence for the operation of the absorption mechanism here postulated would result from the isolation from excised tomato roots of a sucrose phosphorylase or from the demonstration that these roots are capable of rapidly absorbing and dephosphorylating a hexose phosphate. Investigations along these lines are now proceeding.

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SUMMARY

1. Excised tomato roots are sensitive to the initial pH of the culture medium. Good growth is only obtained with initial pH values within the range 4·2 to 5·0 and there is a clearly defined optimum pH at 4·8 to 4·9.

2. The growth of excised tomato roots causes an alkaline drift in the pH of the culture medium and this factor contributes to the decrease in growth rate which occurs with time.

3. Excised tomato roots have a low requirement for inorganic phosphate. The symptoms of phosphorus-deficiency are similar to those associated with carbohydrate deficiency. A rapid restoration of the sucrose absorbing ability of phosphorus-deficient roots results from an external supply of phosphate.

4. Phloridzin inhibits the growth of excised tomato roots. No similar inhibition is observed using the attached seedling root. The phloridzin inhibition of excised root growth is reversible and proportional to the concentration of phloridzin used and is partially counteracted by increasing the sucrose concentration.

5. Excised tomato roots readily utilize sucrose as a carbon source. Dextrose is only able to support a very slow rate of growth and yields roots showing symptoms of carbohydrate deficiency. Galactose, mannose, arabinose, xylose, rhamnose, laevulose, maltose, and raffinose cannot be utilized as carbon sources.

6. Laevulose, galactose, xylose, and maltose are markedly toxic to excised roots simultaneously supplied with sucrose. Dextrose appears to act as a competitive inhibitor of sucrose utilization.

7. Two per cent. sucrose was superior to the other concentrations tested. Symptoms of carbohydrate deficiency were apparent when the concentration of sucrose was reduced to 0·5 per cent. A concentration of 4 per cent. sucrose or higher caused a marked check in growth rate. Using raffinose, which exerts no deleterious effect on the growth of roots simultaneously supplied with sucrose, it was possible to show that the growth obtained with 1 per cent. sucrose can be enhanced by raising the osmotic pressure of the medium and that the unfavourable effect of media of high sucrose content is not a consequence of their higher osmotic pressures.

8. A discussion is presented of the bearing of the above data on the hypothesis that the mechanism of sucrose utilization by excised tomato roots involves its phosphorolysis at the surface of the absorbing cells.

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Inequality in Size of the Male Nuclei in the Genus *Ephedra*

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With Plates IX and X and one Figure in the Text

INTRODUCTION

IT has been previously stated that the two male nuclei in *E. saxatilis* Royle are unequal, the one in front in the pollen tube being smaller, followed in the rear by the larger one (Mehra, 1938). In the same paper the male nuclei in *E. foliata* Boiss. were described as equal. In a later communication dealing with the karyotypes in some species of the genus, *E. altissima* Desf. and *E. intermedia* Shrenk. et Mey. were again described as possessing unequal male nuclei, the male nucleus towards the stalk nucleus end of the grain being invariably bigger than the other in both the species (Mehra, 1947). The inequality in size of male nuclei is also known in the other genera of the Gnetales. Thus Pearson (1929) found unequal male nuclei in *Welwitschia* and the same is known to be the case in some *Gnetum* species. Unequal male nuclei are not confined only to Gnetales. In Coniferales Ferguson (1904) reported unequal male nuclei in *Pinus strobus*. While unequal male nuclei are thus recorded from a number of gymnosperms it is not known what determines this inequality in size. Chamberlain (1935, p. 319) stated: 'In Gymnosperms, in many cases the two sperms differ in size but whether they differ in chromatin content or in any other way, has not been determined either in Gymnosperms or in any other group.' The present study was undertaken to inquire into this problem.

E. foliata Boiss., *E. altissima* Desf., *E. intermedia* Shrenk. et Mey., *E. saxatilis* Royle, *E. sinica* Stapf, and *E. likiagensis* Florin form the subject of the present investigation.

The pollen grains in each case were germinated artificially on glass slides in the natural mucilage secretion that oozes out of micropylar canals of ripe receptive ovules in a manner already described (Mehra, 1947). The fixatives employed were Flemming's weak and Allen's modification P.F.B.$_{15}$ of Bouin's fluid and staining was done in Haidenhain's iron-alum haematoxylin. Besides the facts dealing with mitosis of the body-nucleus leading up to the formation of male nuclei, a study of the cytoplasmic inclusions in the male gametophytes has also been undertaken. The fixatives employed in this latter study are mentioned at the proper places in the text.

[Annals of Botany, N.S. Vol. XIV, No. 55, July, 1950.]

OBSERVATIONS

It is well known that the ripe pollen grain in *Ephedra* consists of two evanescent vegetative cells towards one pole, a large tube nucleus towards the opposite end, and a circular body-cell in the centre on whose periphery is embedded the stalk-nucleus. The position of the stalk-nucleus as a rule is along the long axis of the grain towards the vegetative end, but this is not consistently the case. In many cases the stalk-nucleus may lie on the oblique axis or even along the transverse axis at the periphery of the body-cell. The body-cell cytoplasm is very dense compared to that of the rest of the grain outside it and is bounded externally by a thin membrane. There are invariably embedded in it a large number of small circular bodies, designated as 'spherules' in the body of this paper, which glisten in the living pollen grains in reflected light. The cytoplasm of the gametophyte outside the body-cell is vacuolated and the characteristic 'spherules' are absent.

The body-nucleus is somewhat larger than the stalk-nucleus and like it is highly compact and dense. The entire space is occupied by chromosomes occurring in the form of a knot with very little karyoplasm. Even in the best fixed preparations it is impossible to make out the internal structure of the individual chromosomes, but it can be stated that they are condensed as in an interkinetic nucleus.

As soon as the grains are put on the mucilage secretion they absorb the medium, swell in size, and the prophasic changes start in the body-nucleus. It grows considerably in size and the individual chromosomes elongate. In the mid-prophase their primary constrictions are visible and also their dual nature. It is observed that they are polarized, with the primary constrictions of all facing the end opposite the stalk-nucleus, which means that the chromosomes have undergone no change in their orientation after the previous division. At late prophase the two chromonemata of every chromosome are observed to be interconnected by a few pale staining processes traversing the intra-chromonematal matrix.

About this time the exine ruptures by two longitudinal fissures starting from the tube-nucleus end of the grain and the spindle-shaped gametophyte bounded by the intine jerks out into the germinating medium while the exine undergoes torsion.

On the dissolution of the nuclear wall at the end of the prophase the chromosomes emerge in the form of a polarized bouquet with the free ends of the chromosomes pointing towards the stalk-nucleus (Pl. I, Figs. 1, 2). Evidently the chromosomes have retained the same disposition as at the end of the previous telophase during the period of interkinesis. The chromosomal knot now gradually loosens as the arms spread out and at the same time spindle-fibres are organized towards the two ends, making the position of the kinetochore regions of the chromosomes as the equator. The portion of the spindle towards the tube-nucleus end is the first to be observed (Pl. I, Fig. 5) and is organized from the cytoplasm of the body-cell exclusively, while the spindle-half towards the opposite end is late in appearance and seems to be formed in part at least from the karyoplasm.

The spindle-axis thus incidentally points towards the stalk-nucleus. If the latter lies on the longitudinal axis of the grain, the spindle also assumes the same position, while if it lies on the oblique axis or transversely the spindle-axis follows likewise (Pl. IX, Figs. 3, 6, 8, 9). Apparently, therefore, it appears as if the stalk-nucleus governs the orientation of the spindle-axis of the dividing body-nucleus, but in reality it is because the chromosomes organizing the body-nucleus have not undergone a different orientation from their previous telophasic position, have reappeared in the same fashion, and the spindle is organized in such a way that the position of their primary constrictions becomes its equatorial region.

In *E. foliata* and *E. sinica* the spindle is homopolar, i.e. the two ends of the poles are similarly converging. On the other hand, in *E. altissima*, *E. intermedia*, and *E. saxatilis* the spindle is heteropolar with stalk-nucleus end flat and truncate while the opposite end converges to a point. It is this heteropolar nature of the spindle that determines the inequality in size of the male gametes in these species, as will be soon clear.

At the equatorial plate the daughter chromosomes split and move towards either pole led by their kinetic bodies. In some preparations a centromere granule at the region of primary constriction in the chromosomes at this stage is very clearly observed.

In one case in *E. sinica* a fragment is seen cast off the spindle region while the chromosomes are at the equatorial plate (Pl. X, Fig. 11). How this has come to be it is not possible to say.

The internal structure of the daughter chromosomes at mid-anaphase is not very clear, but there are indications of the presence of two chromonemata, particularly near the free arms. At the late anaphase the spindle elongates, simultaneously undergoing a lateral contraction so that the daughter-chromosome groups are pulled apart. Not infrequently the two arms of a long chromosome may still be joined, forming an equatorial bridge (Pl. IX, Fig. 8 upper, and Pl. X, Fig. 13). The spiral nature of the chromosomes is very obvious in certain preparations and it is observed that the chromonema is certainly not continuous.

At telophase the individual chromosomes undergo further contraction by spiralization. Where the separation of a chromosome arm is delayed, the latter is still observed projecting out from the otherwise smooth contour of the chromosomal clump (Pl. X, Fig. 12).

Formation of the male nuclei

In species with heteropolar spindle the condition of the chromosomes when they arrive at the two poles is different. At the flat truncate pole (facing the stalk-nucleus) the chromosomes, for obvious reasons, remain rather scattered with their free arms spreading (Pl. IX, Fig. 9 left, and Pl. X, Fig. 13). The opposite pole being converging, the chromosomes almost reach the same point and are thus relatively more compacted.

When the nuclear wall is organized around the two groups of chromosomes, obviously the size of the nucleus at the flat end of the spindle where rather scattered chromosomes are being enclosed is greater than the one on the opposite end (Pl. IX, Figs. 6, 7, and Pl. X, Fig. 10). Thus there is no difference whatsoever in the chromatin content of the two nuclei, the inequality being determined by the more dispersed nature of the chromosomes at one end than at the other, which in turn is determined by the heteropolar nature of the spindle. But the amount of karyolymph in the larger nucleus is greater than in the smaller.

In *E. foliata* and *E. sinica* where the spindle is homopolar the two male nuclei formed are equal in size. This is anticipated even at telophase, where the chromosomal knots at two poles are observed to be of the same size (Pl. X, Fig. 4, and Pl. IX, Fig. 12).

During the formation of the male nuclei the chromosomes loose their staining capacity gradually and in proportion to this the light-grey substratum in which they are embedded assumes a pale appearance. It seems as if some substance is secreted out of the chromosomes which on reacting with the surrounding medium changes its staining reaction from greyish to pale. The pale substance is the karyolymph and around it is deposited the nuclear wall.

The male nuclei then gradually increase in size. In species where they are unequal the same proportional difference in size is maintained during their growth.

During the growth of the male nuclei the chromosomes, which had previously undergone a high degree of contraction due to spiralization at telophase, also lengthen out. Although they stain feebly, yet their outlines are clearly visible. The chromonema threads of these is not continuous, but there are gaps at intervals.

After the organization of the male nuclei the spindle disappears, starting from the polar ends (Pl. IX, Figs. 6, 7).

E. altissima is a tetraploid and produces besides the normal $2n$ pollen a small percentage of $4n$ pollen (Mehra, 1947). The two male nuclei in the normal pollen are unequal. Pl. X, Fig. 10, shows a giant $4n$ pollen in the same species, and the male nucleus facing the stalk-nucleus is observed to be bigger than the other as in the normal grain. Similar size differences in the two nuclei are maintained in the tetraploid pollen of *E. saxatilis* and *E. intermedia*, which are characterized by the inequality in size of the male nuclei in their normal $2n$ pollen.

In *E. likiagensis* the male nuclei may be equal or somewhat unequal with none of the exaggerated difference in size observed in *E. altissima*, *E. intermedia*, and in particular *E. saxatilis*.

At the end of the formation of the male nuclei the pollen tubes are put out, the details of which have already been described (Mehra, 1938).

In artificial culture and also in the pollen tube developed normally in the pollen chamber in the ovule in *E. saxatilis* and *E. intermedia* it is observed that the smaller gamete always precedes the larger one (unpublished data), and this is presumably the one involved in fertilization.

DURATION OF THE MITOTIC CYCLE

The duration of the mitotic cycle varies with the nature of the medium in which the germination of the pollen occurs and the temperature. In natural mucilage secretion the time taken is much less than in any concentration of saccharose solution (Mehra, 1938).

It takes approximately 5¼ hours for complete mitosis to be effected in natural mucilage at a temperature of 92–3° F. in *E. foliata*, while this is reduced to 3 hours only at 96–8° F.

The duration of each mitotic stage was determined for *E. foliata* pollen germinated on natural mucilage secretion at 92–3° F. The culture was started at 11 a.m. and the stages in division were directly observed under the microscope at short intervals. These were further confirmed by staining a test sample with acetic-iodine green.

The prophase lasted for 2 hours (till 1 p.m.). The spindle was organized and the chromosomes assumed their position in the form of an equatorial plate by 2.37 p.m., thus taking 1 hour and 37 minutes. The movement of the daughter chromosomes to the two poles was effected in only 1 hour and 13 minutes (by 3.50 p.m.). It took another 15 minutes for the male nuclei to be organized. The total period of mitosis thus lasted for a little over 5 hours at 92–3° F.

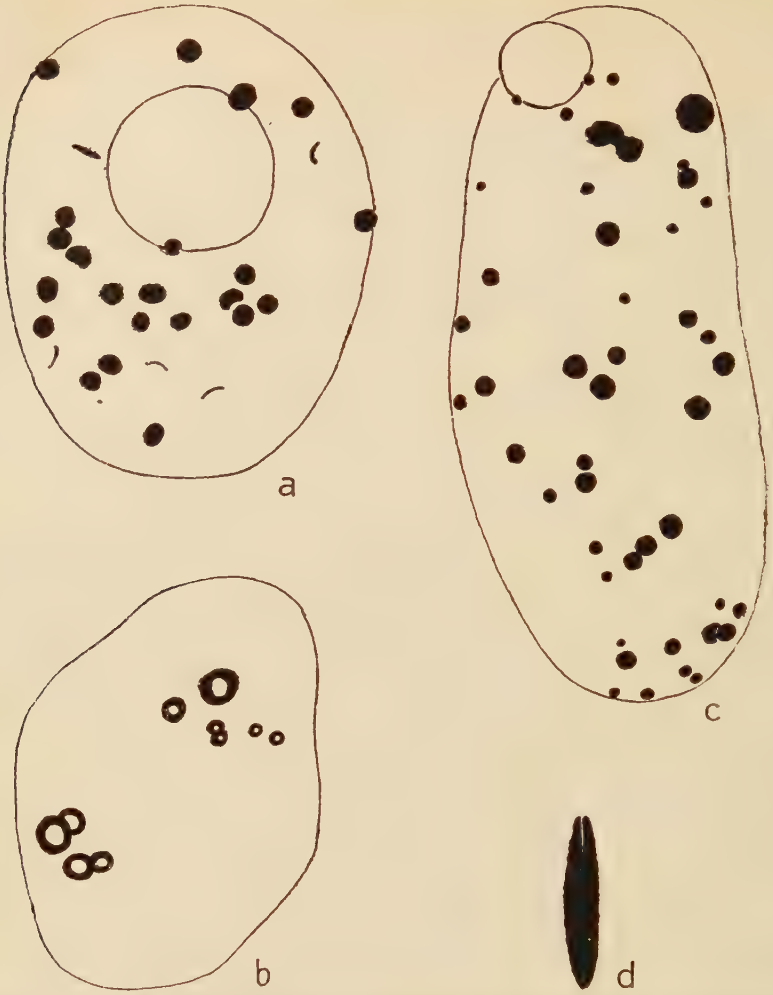
CYTOPLASMIC INCLUSIONS

It has already been stated that the cytoplasm of the body-cell is very dense and is devoid of any vacuoles. In it are present two types of bodies:

1. Small spheres or 'spherules' which occur invariably.
2. Spindle-shaped bodies or 'spindlets' which are of sporadic occurrence.

Spherules

These are of invariable occurrence in the body-cell cytoplasm of all the six species of the genus under investigation and their presence must therefore be regarded as a rule in the genus (Pl. X, Figs. 13–16, and Text-fig. 1 *a* and *c*). Outside the body-cell cytoplasm they are absent. Because of their high refractive index compared to the cytoplasm in which they are embedded, they appear as highly glistening bodies in reflected light under high-power or oil-immersion lens in the living pollen grains, particularly when the exine is cast off during their germination. In size the spherules vary from mere dots to those of considerable size. Their number in a body-cell varies in accordance with their size. When dot-like they are quite numerous, but when of bigger size they are comparatively fewer. In a body-cell all the spherules may be dot-like or there may be a slight variation in size or the majority may be of uniformly large size with a few dot-like intermixed, or in still other cases there may be a good deal of variation in size (Text-fig. 1 *c*). The larger of these spherules



TEXT-FIG. 1. *a*. *E. foliata*, body-cell showing the entire spherule and spindlet material. The body-nucleus is shown in outline. From living material × 1,840. *b*. *E. foliata*, body-cell from a living gametophyte after it had been mounted in water for 30 minutes. Spherules have considerably swollen and their vacuolous nature intensified × 1,840. *c*. *E. altissima*, body-cell fixed in Flemming's W.R.T. for *Gasteria* and stained in iron-alum haematoxylin. Stalk-nucleus shown in outline and the body-nucleus which is at metaphase not drawn. Entire spherule material shown × 1,840. *d*. Spindlet from preparation shown in fig. 17 of Pl. II. Highly magnified.

are particularly refractile in the living condition. They may be irregularly scattered singly or in several irregular groups. Often they form beautiful chains (Pl. X, Figs. 14, 15), but all these arrangements are merely incidental. During the division of the body-nucleus they are never present in the spindle zone, and when the two male nuclei are organized the spherules may be aggregated round them, but this is of no special significance.

The spherules are particularly susceptible to fixatives containing acetic acid and are therefore lipoidal in nature. In Bouin's fixative they never appear (Pl. IX, Figs. 1–4, 8, 9). Beautiful preparations are, however, obtained after fixation in Flemming's for *Gasteria* with only traces of acetic acid followed by staining in iron-alum haematoxylin (Pl. X, Figs. 13–15), or when Flemming's weak fixative with traces of acetic acid is used with subsequent staining in iron-alum haematoxylin.

The spherules are undoubtedly vesicular in nature. This is observed in the larger spherules in the living condition when the outer rim appears dense and more refractive than the interior. In fixed preparations stained with iron-alum haematoxylin and properly differentiated the same thing is observed. If the living grains are immersed for a period of half an hour in ordinary water, the cytoplasm of the gametophytes gradually undergoes a process of senescence, but during this period some of the larger spherules swell considerably in size and their vesicular nature becomes quite conspicuous (Text-fig. 1 *b*).

In fixed preparations often two or more spherules are observed fused together into an irregular mass (Text-fig. 1 *c*). Whether or not this has something to do with the action of fixative on closely situated spherules is not clear. This seems possible in view of the fact that under the action of the fixative the spherules often loose their perfectly spherical outline and become hemispherical or lunar or develop angularities.

The total spherule material in the body-cell of *E. foliata* is about half of that in the other five species. This is in accordance with the size of the pollen grains in the two cases, which in turn is governed by the fact that the pollen grains in *E. foliata* possess a set of seven chromosomes while those in *E. intermedia*, *E. sinica*, *E. likiagensis*, *E. altissima*, and *E. saxatilis* each possess a set of fourteen chromosomes (Mehra, 1947).

Again it has been found that in *E. intermedia*, *E. altissima*, and *E. saxatilis* besides the normal grains with fourteen chromosomes a small percentage of giant grains with twenty-eight chromosomes is also met with (Mehra, loc. cit.). The spherule material in such giant grains is again roughly double to that met with in the normal grains of these species.

It has been stated that spherules are lipoidal in nature. Fixatives containing acetic acid do not preserve them. If a drop of dilute acetic acid is run under the coverslip containing fresh, living, germinating pollen grains underneath, the glistening spherules immediately disappear. They are Feulgen-negative and give negative tests for starch, proteins, oils, and fats.

It is difficult to state the exact nature of spherules. They correspond most closely to the microsomes or lipide granules of Guilliermond (1941), but there is a distinctive difference in that the spherules are vacuolar whereas the microsomes are not. They are not the mitochondria because of their much larger size, their highly refractive nature, and their inability to take the intravitem stain Janus green, although they resemble the mitochondria in being vacuolar as the latter sometimes are (Guilliermond, loc. cit.). Also with fresh coverslip osmication in 2 per cent. solution of osmic acid for 20 hours they take only a very feeble stain, whereas the mitochondria stain jet black. They most certainly do not belong to the category of either plastids or vacuome.

Spindlets

These do not seem to be of constant occurrence in the body-cell cytoplasm. In the material fixed in Flemming's W.R.T. for *Gasteria* and stained in iron-alum haematoxylin they may be observed in some grains while they are absent in others.

When young these bodies are in the form of straight or slightly curved threads (Pl. II, Fig. 16, and Text-fig. 1 *a*), but they grow in size and when mature appear in the form of short spindlets. Often the two spindlets may be observed in such close approximation with each other along their longitudinal axis that it gives the impression that they multiply by longitudinal fission. In Text-fig. 1 *d*, drawn from the preparation shown in Pl. X, Fig. 17, a fairly mature spindlet is observed which appears to be in an early stage of fission, as indicated by the non-staining line in the middle at one of the poles. This is not so clear in the photograph.

The number of spindlets when present are variable. There may be just one large spindlet in the entire body-cell or there may be three, four, or more relatively smaller ones (Pl. X, Figs. 17, 16 respectively). The distribution of these spindlets, like the spherules, is irregular.

The nature of these spindlets or their function is not known. Whether they are of the nature of crystals is problematical.

The cytoplasm outside the body-cell is thin and highly vacuolated. There are no spherules or spindlets present. With very dilute intravitem neutral red solution the vacuoles take reddish coloration and soon their colloids are precipitated in the form of small granules which perform rapid Brownian movements.

SUMMARY

The development of male nuclei from the division of the body-cell nucleus has been studied in six species of the genus *Ephedra*.

In *E. foliata* and *E. sinica* the two male nuclei are of the same size, in *E. likiagensis* the male nuclei may be equal or unequal, but in the latter case there is no marked difference, while in *E. altissima*, *E. intermedia*, and *E. saxatilis* they are markedly unequal, particularly in the last species.

The inequality, where present, is due to the heteropolar nature of spindle of the dividing body-nucleus. The spindle-half towards the stalk-nucleus end is late in appearance, truncate and blunt at the pole, in contrast to the spindle-half on the other side which is converging. The chromosomes when they reach the pole at the blunt end are consequently spread compared to those on the opposite side where they form a relatively compact mass at telophase. The nucleus formed towards the stalk-nucleus end, therefore, is invariably bigger than its sister on the other end. There is thus no difference in the amount of chromatin in the two nuclei, but there is obviously some in the amount of karyolymph. The two male nuclei, if unequal, maintain this difference even during their further growth.

Species which possess unequal male nuclei in the x pollen grains exhibit the same difference even in the $2x$ ones if present.

There are invariably present in the dense cytoplasm of the body-cell spherical bodies vasicular in nature which glisten in reflected light in fresh condition. Their chemical nature is lipoidal.

In addition there occurs sporadically in the body-cell cytoplasm another type of body here called 'spindlets'. When young they are in the form of straight or slightly curved threads of short lengths which on maturity assume spindle-shaped outline.

The cytoplasm outside the body-cell is vacuolated and does not contain any of the two types of bodies stated above.

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EXPLANATION OF PLATES

Illustrating P. N. Mehra's article 'Inequality in Size of the Male Nuclei in the Genus *Ephedra*'. All figures from untouched photographs.

Figs. 1–12 from preparations fixed in Allen's modification P.F.B.$_{15}$ of Bouin's fixative. Figs. 13–17 from preparations fixed in Flemming's W.R.T. for *Gasteria* with only traces of acetic acid. In all cases the exine has been cast off.

PLATE IX

Figs. 1–4. *E. foliata.*

Figs. 5–7. *E. intermedia.* Mark in Fig. 5 the converging spindle-half on the side opposite the stalk-nucleus, while the one towards the stalk-nucleus end is not yet organized. In Figs. 6 and 7 unequal male nuclei are observed.

Figs. 8–9. *E. saxatilis.*

PLATE X

Fig. 10. *E. altissima.* A $4n$ pollen grain showing unequal male nuclei.

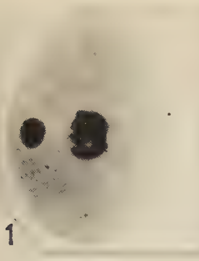
Figs. 11–12. *E. sinica.* Mark the equal male nuclei in Fig. 12.

Figs. 13–14. *E. saxatilis*, showing the spherules.

Fig. 15. *E. intermedia.* Only spherules are present and no spindlets.

Fig. 16. *E. intermedia*, showing both spherules and spindlets.

Fig. 17. *E. intermedia.* A big spindlet is observed with a few spherules, the remaining spherules being out of focus.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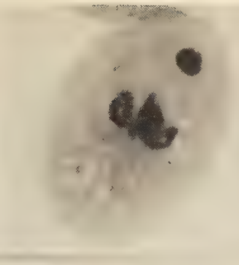
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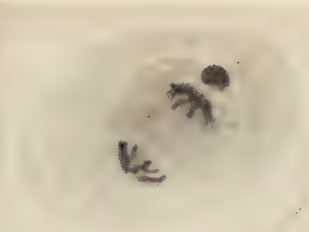
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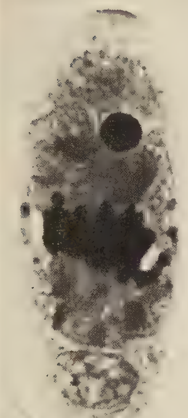
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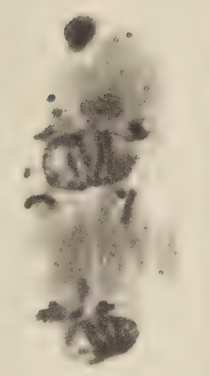
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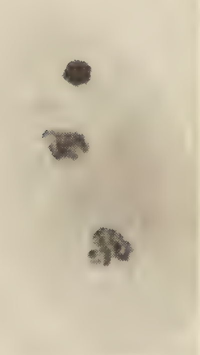
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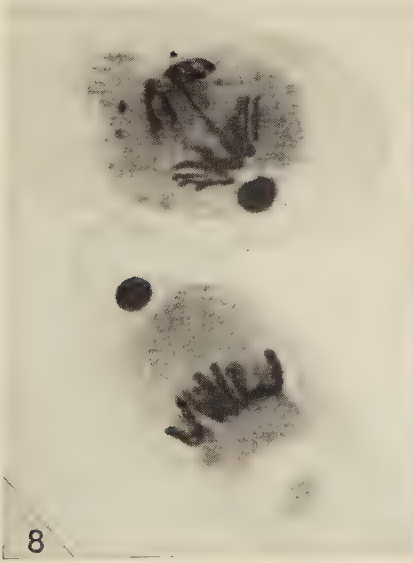
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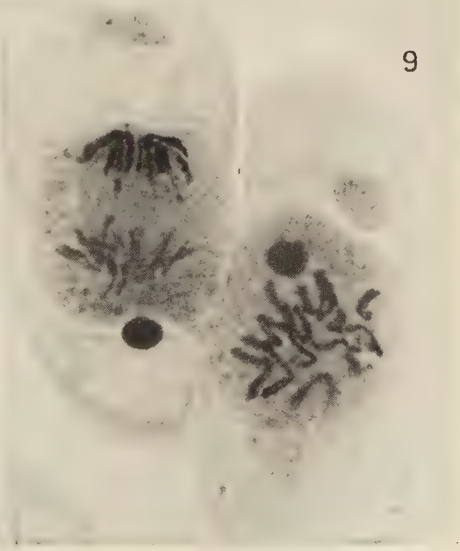
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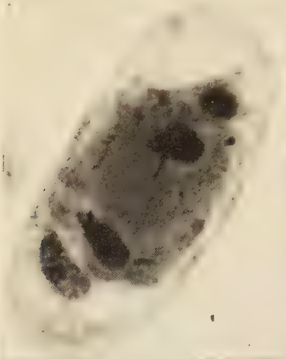
P. N. MEHRA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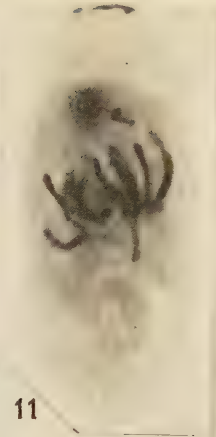
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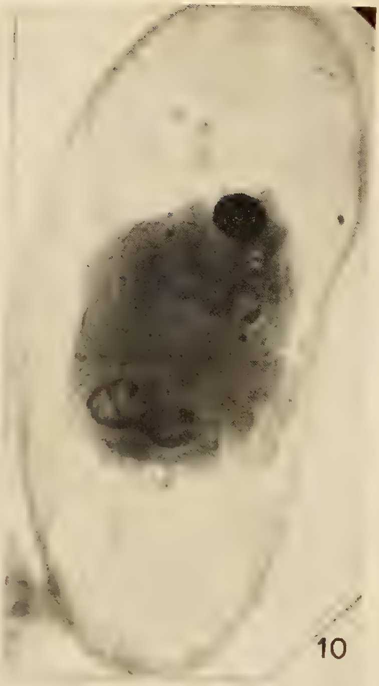
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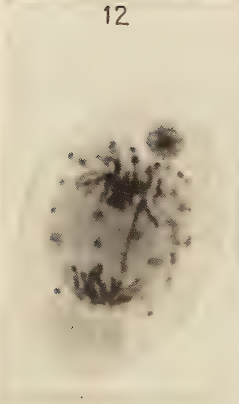
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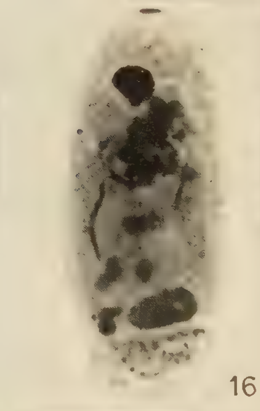
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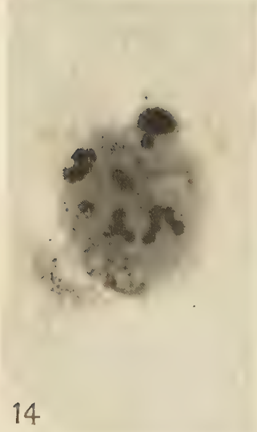
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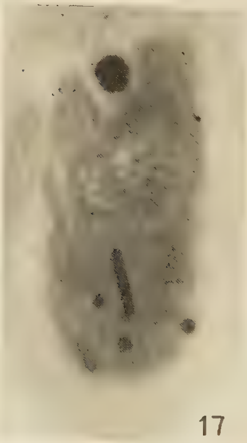
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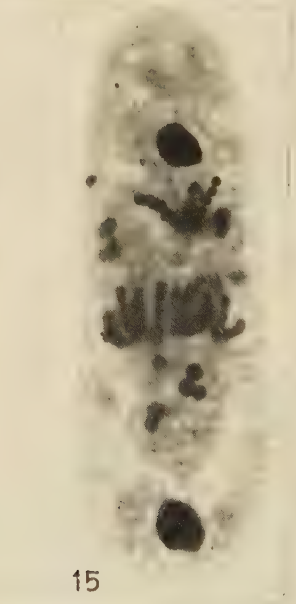
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P. N. MEHRA

Alternation of Generations and Heteroecism in *Puccinia versicolor*

BY

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With Plate XI and 1 Figure in the Text

INTRODUCTION

HETEROPOGON contortus (L.) Beauv., commonly known as spear-grass, is widely distributed through the tropics and subtropics. It is a common grass of the dry pasture-lands in south India. *Puccinia versicolor* Diet. and Holway is also widely distributed on this grass in India. A detailed field and cultural study has revealed that the rust is heteroecious with pycnial and aecial stages on *Canthium parviflorum* Lam. (*Plectronia parviflora* Bedd.), a common shrub of the dry waste lands.

MATERIAL AND METHODS

Material for spore germination and inoculation studies was collected in Bangalore during different seasons and stored in paper bags at room temperature. The aecio-, uredio-, and teliospores were germinated on slides by adopting the technique suggested by Thirumalachar (1940).

For inoculation experiments the plants were grown in pots under rust-free conditions and the usual methods of inoculation were followed. For cytological studies the material was fixed in formalin acetic alcohol or Allen's modification of Bouin's fluid, and microtome sections of 8 to 10μ thickness were cut and stained with Heidenhain's iron-alum haematoxylin or Newton's iodine gentian violet.

OBSERVATIONS

Life-cycle on Heteropogon contortus

Uredia

Morphological details of the uredia and telia are already given by Dietel and Holway (1897), the Sydows (1904), and Doidge (1926). The uredia first appear on the spear-grass in July–August, developing on reddish-yellow spots. The sori are yellowish-white, erumpent and pulverulent. The uredio-spores are ovate-globose, hyaline, and with an epispore which is echinulate and irregularly thickened to impart a stellate appearance in the interior

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(Text-figs. 1 and 2). The germ-pores are indistinct and become apparent only during germination.

The urediospores readily germinate in water, developing long germ-tubes (Text-fig. 3). Spores from freshly erupted sori when placed for germination on slides at room temperature (about 24° C.) gave between 90 and 100 per cent. germination. Viability of the urediospores in material stored under laboratory conditions showed that it decreased gradually and no viable spores were observed after 30 days.

The urediospores incite secondary infection of the spear-grass and there is a rapid spread of the rust in the field from August to November. After the development of the telia, secondary uredia are no longer produced. The host plants begin to dry up by the end of April, and in May and the early part of June no viable urediospore material can be collected in the field. The question of over-summering of the rust in the uredial stage is of secondary importance, since the uredial stage is easily initiated by aeciospore infections from the alternate host.

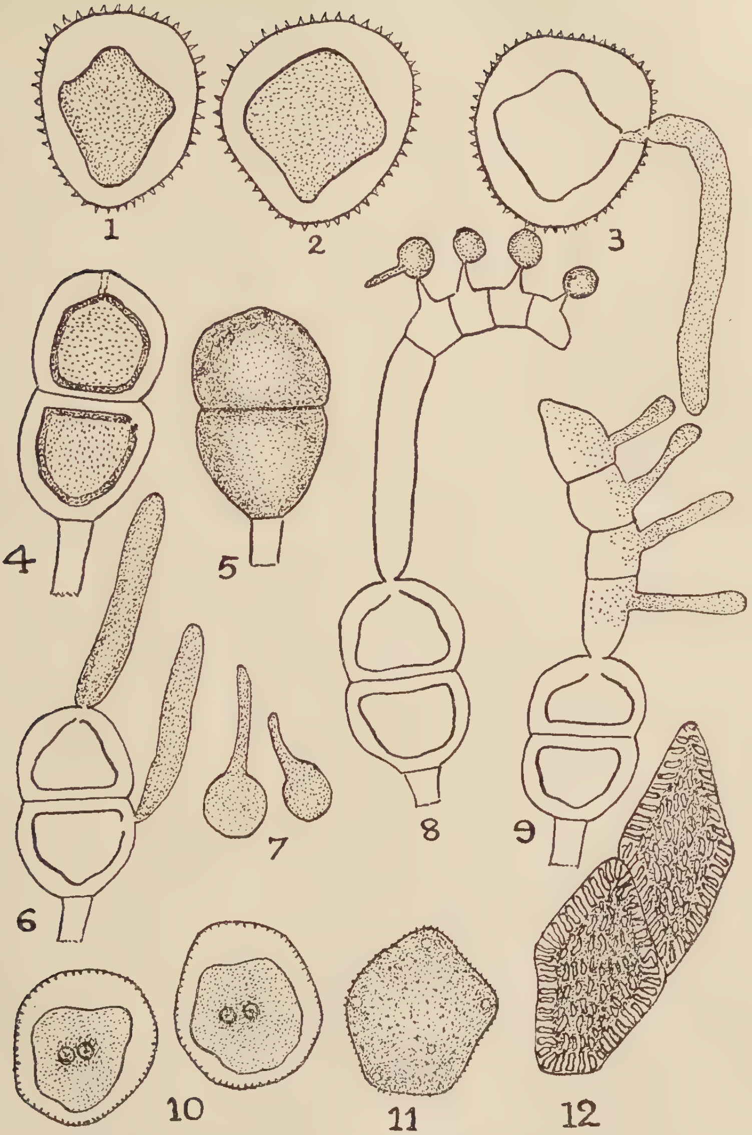
Inoculation experiments were carried out using urediospores. Spear-grass taken from clumps growing in rust-free areas or from seedlings were inoculated. The development of secondary uredia were noticed after 15 to 20 days. Doidge (1926) reports *Themeda triandra* Forsk., *Hyparrhenia* sp., and *Monocymbium ceresiaeforme* Stapf as additional grass hosts for *Puccinia versicolor*. No cross inoculations using urediospores were carried out in the present study to check the host range.

Telia

Telia follow uredia in development in December, appearing as tiny black specks. In April and May, when the production of secondary uredia has ceased, the telia can be collected in abundance. In early stages the teliospores are developed within the uredia and later completely replace them. Mature teliospores are reddish-brown, oblong-ellipsoid, slightly or not constricted at the septa (Text-figs. 4 and 5), and smooth. The walls are up to $2{\cdot}5\,\mu$ thick and possess a single germ-spore in each cell. Cytological studies indicate that the young spores are binucleate, the two nuclei fusing in the mature spore.

The teliospores have been germinated in large numbers. Spores from freshly collected material in December and January yielded very poor results. Not more than 5 per cent. of the spores were seen to germinate after 5 to 6 days in moist chambers. The teliospore material collected in March was divided into two lots, one spread in shade in the field on April 1, 1949, and thus exposed to natural environmental conditions, the other lot stored in the laboratory at room temperature. The temperature in shade between April 1 and May 15, 1949, in Bangalore showed an average minimum of about 70–3° F. and an average maximum of about 93–5° F.

Germination tests were carried out at various intervals during this period. The percentage of teliospore germination was very low during April and gradually increased towards the middle of May. In tests carried out on May 15



Text-figs. 1–12. Figs. 1 and 2. Urediospores (× 800). Fig. 3. Germinating urediospores (× 800). Figs. 4 and 5. Teliospores (× 750). Fig. 6. Early stage in teliospore germination (× 750). Fig. 7. Germinating sporidia (× 750). Fig. 8. Germinating teliospore bearing sporidia (× 750). Fig. 9. Abnormal teliospore germination (× 750). Figs. 10 and 11. Aeciospores (× 800). Fig. 12. Peridial cells (× 1,000).

the teliospores from material exposed to natural environmental conditions germinated 80 to 100 per cent., the sporidia being formed within 10 hours. In contrast, the teliospores in material stored in the laboratory during the same period showed very poor germination, not amounting to more than 4 per cent.

Teliospores from material after-ripened in the field germinated readily when placed in moist chambers. Both cells of the teliospore germinate (Text-fig. 6), producing a four-celled promycelium (Text-fig. 8). The sporidia are globular, hyaline, and germinate *in situ*, developing long germ-tubes (Fig. 7) or occasionally secondary sporidia. In stained preparations the sporidia show a single nucleus. Abnormal teliospore germinations due to excess of moisture have been noticed in several instances. The sterigmata in such cases elongate without sporidial formation (Text-fig. 9).

Life-cycle on Canthium parviflorum

The existence of an alternate host bearing the pycnial and aecial stages became evident after intensive field studies for several seasons. In July the uredial stage usually appeared first on clumps of spear-grass near *Canthium parviflorum*, a thorny shrub belonging to the Rubiaceae. An unconnected *Aecidium* closely agreeing with *Aecidium plectroniae* Cooke has been collected by the writers for several years. The appearance of the aecial stage on *Canthium* in the lawns following the monsoon rains in July was a strong indication that it was possibly connected with some grass rust.

In order to confirm this suspected heteroecious cycle of *Puccinia versicolor*, after-ripened teliospores were germinated on slides and used for inoculating young unfolding leaves of *Canthium parviflorum* grown in the laboratory under rust-free conditions. The inoculated plants were enclosed in moist chambers for 48 hours, after which they were placed outside for further observation. As many as sixty-five separate inoculations were carried out and all yielded successful infection. Several controls all remained healthy.

The first symptom following successful infection became evident after 8 days by the development of swollen pale greenish patches on the young leaves. The infection causes marked hypertrophy. Pycnia, which are amphigenous, appear first as greenish viscid specks. Sections through the leaf indicate that they are subepidermal, angular, deep seated, with few ostiolar paraphyses (Pl. XI, A). The aecia follow the pycnia in development after a period of 12 to 18 days (Pl. XI, C and D). They are mostly hypophyllous, erumpent, and pulverulent, white, with well-developed peridia (Pl. XI, B). The cells of the peridia are firm, thick-walled, polygonal to rhomboidal with folded rugose thickenings (Text-fig. 12). The aeciospores are white in mass, developed in chains, angular to polygonal, slightly thickened at the apex, and possess hyaline contents (Text-fig. 10). The epispore is pitted, presenting a verruculose appearance and possessing 6 to 7 scattered indistinct germ-pores (Text-fig. 11). In stained preparations the young pycnia and aecia are evidently haploid. The aeciospores are binucleate, but the initiation of the dicaryophase was not observed.

Aeciospores from freshly erupted sori germinate with long germ-tubes within 24 hours when placed in moist chambers. Such spores were used for inoculating spear-grass plants, which were enclosed in moist chambers for 48 hours and then transferred to a greenhouse for further observation. Evidence of successful infection became manifest after 10 days by the appearance of reddish-yellow specks on the blades of spear-grass. The development of uredia took place 15 to 20 days after inoculation and thus completed the cycle of development.

Discussion and Summary

The heteroecious nature of *Puccinia versicolor* was first inferred from field studies and later confirmed by cross-inoculation experiments. The aecial stage on *Canthium parviflorum* was produced in the laboratory nearly 2 months in advance of its natural appearance in the field.

As regards the identity of the aecial stage, no less than nine unconnected *Aecidium* states have been described on the host genus *Canthium*. The aecial stage of *Puccinia versicolor* comes nearest to *Aecidium plectroniae* Cooke. No aecial stage has hitherto been recorded on *Canthium* in India.

It is interesting to note that in places where the uredial and telial stages on *Heteropogon contortus* have been reported in India and South Africa, the aecial stage on *Canthium* also occurs in the same locality. Several other *Aecidium* species such as *A. plectoniicola* P. Henn., *A. baumianum* P. Henn., *A. busseanum* P. Henn., *A. baileyanum* Syd., *A. incomparabile* Syd., which are recorded on *Canthium* spp. from different parts of the world, resemble the aecial stage of *Puccinia versicolor* to some extent. Some of these are possibly connected with other rusts on Andropogoneae.

In conclusion the writers wish to express their gratitude to Dr. G. R. Bisby, Commonwealth Mycological Institute, Kew, for suggestions and criticism.

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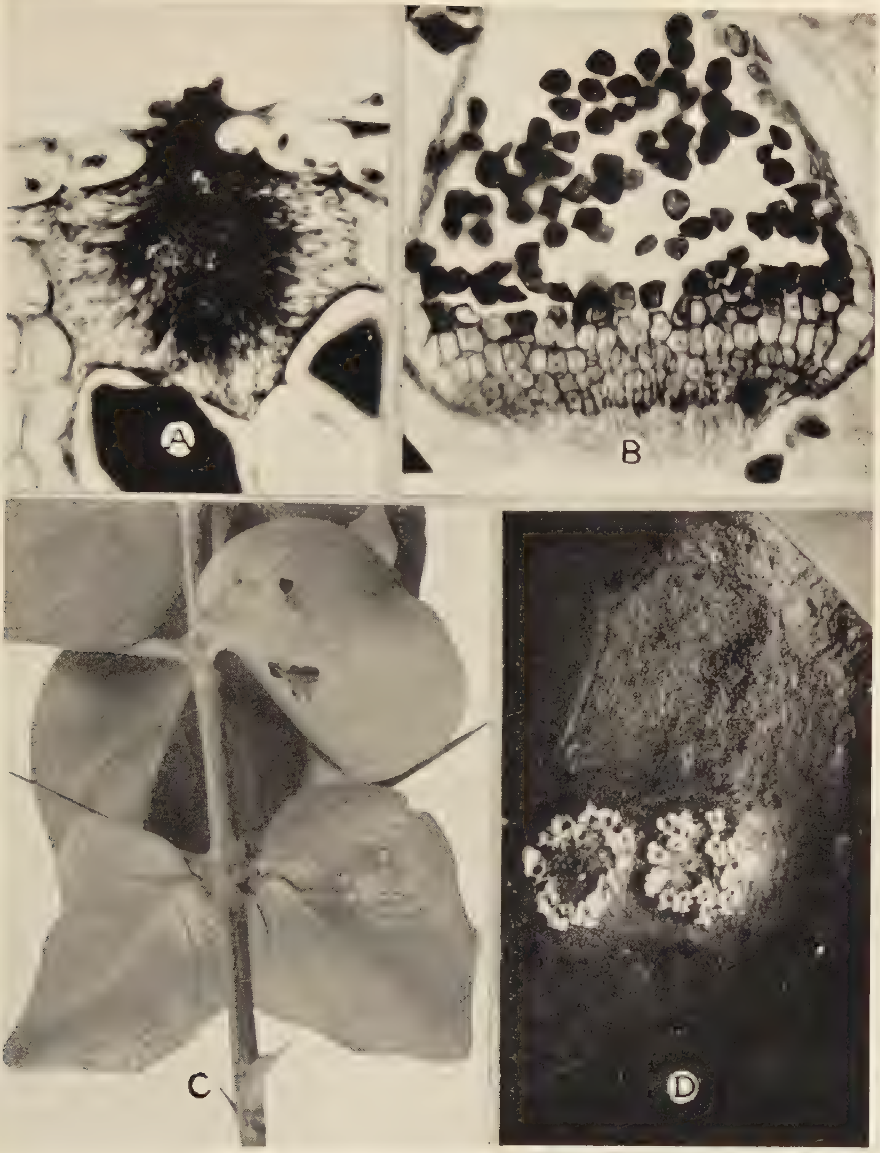
EXPLANATION OF PLATE

Illustrating M. J. Thirumalachar's and M. J. Narasimhan's article 'Alternation of Generations and Heteroecism in *Puccinia versicolor*'

A. Pycnium. ×200.
B. Aecium. ×150.
C. Aecia produced on *Canthium parviflorum* by artificial inoculation. ×1½ nat. size.
D. Enlarged view of the same. ×7.



THIRUMALACHAR and NARASIMHAN

Studies on the Germination of the Seeds of *Striga Hermonthica*

I. The Influence of Moisture-Treatment, Stimulant-Dilution, and After-Ripening on Germination

BY

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With ten Figures in the Text

INTRODUCTION

IT has been known for many years that the optimum germination of the seeds of some angiospermous root parasites will only take place in the presence of some stimulant exuded from the roots of suitable host plants. The effects of moisture-treatment and stimulant-dilution on the germination of the seeds of the root parasites *Orobanche speciosa* and *Striga lutea* have been investigated by Chabrolin (1938) and Brown and Edwards (1944, 1946) respectively. These workers showed that if the seeds of these root parasites were exposed to moisture, prior to the application of a suitable stimulating solution, the rate of germination was enhanced, and also that, as moisture treatment was extended, a stage was reached at which the stimulating solution could be diluted 1,000 times without significantly affecting germination.

The present paper relates to an investigation of the effects of moisture-treatment and stimulant-dilution on the germination of the hemi-parasite *Striga hermonthica*. It was thought best, however, to consider these effects in relation to the possibility that the seeds might require after-ripening in dry storage. In this latter respect the present investigation differs from those of earlier workers.

To enable the possible influence of after-ripening to be investigated, seeds harvested in different years were employed. These seeds were harvested in the Sudan in 1940 and 1941 and in Tanganyika in 1945 and 1948. For supplying this material the author wishes to acknowledge his indebtedness to Dr. F. W. Andrews, the Gezira Research Farm, the Anglo-Egyptian Sudan, and to the Empire Cotton Growing Corporation.

EXPERIMENTAL METHODS

The stimulating solution employed in the germination studies consisted of glass-distilled water in which the roots of *Sorghum vulgare* (Sudan grass), a suitable host plant, had been allowed to grow for a constant period. The

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method of preparation was similar to that of Brown and Edwards (1944). Seeds of *Sorghum vulgare* were allowed to germinate for 2 days at 32° C. Fifty of the resultant seedlings, of approximately equal size, were placed on a raft made from one layer of surgical gauze and a ring of egg-shell glass tubing. This raft was floated on 50 ml. of glass-distilled water inside a staining-jar and the jar sealed by means of a rubber stopper. The jars and their contents were then maintained at 22° C. in an incubator for 5 days. The liquid inside the jars was then ready for use as the stimulating solution. Immediately prior to its use the contents of three jars were mixed, for it was considered that by this means possible variations in the concentration of the stimulating substance would be reduced. It was found necessary to use the mixture immediately, for on exposure to the air it rapidly lost its activity.

The method used for investigating the effects of moisture-treatment and stimulant-dilution on the germination of the seeds of *Striga hermonthica* was adapted from that of Brown and Edwards (1944). It involved two distinct yet complementary treatments. Following the terminology of Brown and Edwards (1944) the first treatment was termed 'pretreatment'. To effect pretreatment the seeds were moistened with distilled water and maintained at a constant temperature for varying periods. The temperature of pretreatment was not the same in all experiments, and altogether seeds were pretreated at temperatures of 12° C., 22° C., 27° C., and 32° C.

After periods of pretreatment varying from 1 to 51 days samples of seed were removed from the incubators. Each sample of seeds was divided into two lots and half were treated with the stimulating solution and half were used for the control series. In the control series distilled water was used instead of the stimulating solution. The seeds exposed to the stimulating solution and those of the control series were then maintained at 32° C. for 48 hours. This latter treatment constituted the second phase of the germination method.

The amount of seed used for any experiment in which the seed required pretreatment was standardized at 0·5 g. For pretreatment the 0·5 g. sample was placed in a 2 × 2 Pyrex sintered glass Gooch crucible which was then put into a 150-ml. Pyrex beaker, and the beaker was filled with glass-distilled water up to the lower level of the sintered disc. After this the beaker and its contents were placed in an incubator maintained at one of the appropriate temperatures mentioned above. Fungal growth inside the crucibles was minimized by washing the seeds with distilled water at the same temperature as the seeds. Samples of seed were removed from the crucibles and were exposed to the stimulating solution immediately after washing. It would have been preferable to have kept the seeds in total darkness throughout the experimental period, but this was not possible. However, the period of exposure of the seeds to daylight was standardized at 5 minutes every 3 days of pretreatment.

The seeds of *Striga hermonthica* are relatively minute (0·4 × 0·2 mm.) and thus microscopical examination is necessary to determine germination. The method of exposing *Striga* seeds to the stimulating solution facilitated microscopic inspection but differed from that of Brown and Edwards (1944) in that it permitted a representative population of seeds to be examined. Small ointment pots with a capacity of 25 ml. were used as the germination vessels. The bottoms of these pots were concave and so before the seeds were placed inside it was necessary to prepare a platform on which the seeds could be sown. With this aim in view a few glass beads were placed in each pot. Two discs of 4·25 cm. filter-paper were laid on top of the beads, and 3 ml. of either the stimulating solution or distilled water were then added to each vessel. After this, a well-washed 4-cm. disc of permeable cellophane was placed on top of the filter-paper. The pots were then ready for use as germination vessels. At this stage 150–200 *Striga* seeds were spread evenly over the cellophane by means of a camel-hair brush.

After sowing, the ointment pots were sealed to prevent water loss. This was accomplished by fixing a disc of water-proof cellophane over the top of each pot and securing it to the lip of the pot by a rubber band. The ointment-pot lids were then replaced and held in position by rubber bands.

For germination counts the cellophane disc together with the attached seeds was removed, then placed on a grid scratched on a glass plate and examined under a binocular microscope.

All germination tests were replicated at least 3 times and all data were analysed for variance.

EXPERIMENTAL RESULTS

Preliminary observations

Preliminary experiments were first carried out in 1946 to determine whether germination could be induced by treating the dry seeds with the stimulating solution. It was found that no more than 0·1 per cent. of the seeds germinated when they were placed in vessels containing the stimulating solution and were then incubated at either 22° C. or 32° C. for varying periods up to 21 days. (During these experiments the water lost by evaporation was replaced every 3 days.) The seeds used in the preliminary experiments were harvested in 1940, 1941, and 1945 respectively. Consequently it seemed unlikely that the failure to induce more than a very small percentage of germination could be attributed to the use of immature seed.

The experiments so far described did not show whether the dry seeds were impermeable to the stimulant, whether the stimulant was having any action on the dry seeds, or whether the stimulant was effective in promoting some reaction in the seed, as a result of which subsequent germination was almost completely inhibited. Further experiments were clearly necessary to determine which, if any, of these three factors was involved when dry seeds were added to the stimulating solution. Additional observations suggested that the dry seeds were not impermeable to the stimulant and that the stimulant did have some very definite action on the seeds which almost entirely prevented subsequent germination. Thus when dry seeds were placed in germination vessels containing the stimulating solution and were transferred to fresh stimulating solution every 3 days only 0·1 per cent. germinated as before. On the other hand, when seeds harvested in 1940 and 1941 were given as little as 24 hours of moisture-treatment at a temperature of 22° C., and were then placed in germination vessels containing the stimulating solution, up to 10 per cent. germinated. It seemed therefore that a short period of moisture-treatment was essential if more than 0·1 per cent. of the seeds were to germinate.

These preliminary experiments showed that in common with the seeds of *Orobanche speciosa* (Chabrolin, 1938) and of *Striga lutea* (Brown and Edwards, 1944, 1946) the germination process of the seeds of *Striga hermonthica* involves two major phases. During the first phase, termed pretreatment, moisture only is required. During the second the stimulant is needed also. In view of the importance of the moisture-treatment, observations were made to determine how far it governs the amount of germination, using seeds of four different ages.

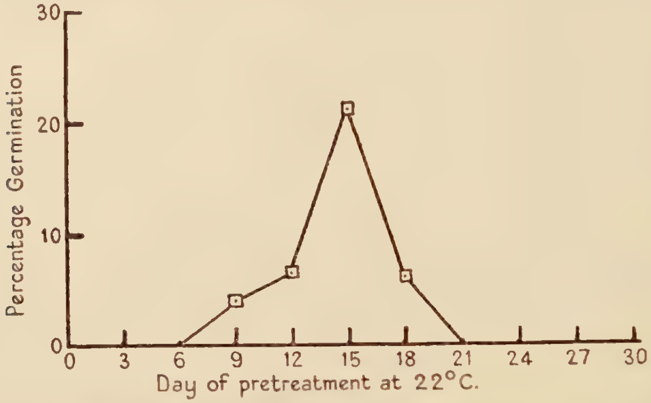

FIG. 1. The relationship between length of pretreatment at 22° C. and the germination capacity of 1945 seed. (Pretreatment initiated April 1946: standard error of each mean germination percentage = ±1·5.)

The effect of pretreatment and after-ripening on germination

When samples of 1945 seed were pretreated at 22° C. in April 1946, the germination capacity of the seed, after standard treatment with the stimulating solution, was found to vary with the length of pretreatment. The results of these experiments are shown in Fig. 1. Essentially similar results were obtained when 1948 seed was pretreated 4 months after harvesting. It may be seen from Fig. 1 that after 6 days of pretreatment at 22° C. germination was less than 1 per cent. From the 6th to the 15th day of pretreatment the percentage germination increased progressively and reached a peak value of only 21 per cent. after 15 days. During the subsequent period of pretreatment the percentage germination decreased, and after 21 days no more seeds germinated.

The low germination capacity, observed at the optimum stage of pretreatment in this experiment, was almost certainly due to immaturity of the seed employed. The results of additional studies on the germination of 1945 seeds, extending over a period of 15 months, confirmed this view. In these experiments samples of 1945 seed were pretreated at 22° C. prior to the application of the stimulant; but the experiments were separated by time-intervals varying from a few days to 6 months. A representative series of the germination curves obtained are shown in Fig. 2, which indicates also the month in which the pretreatment of each sample was initiated.

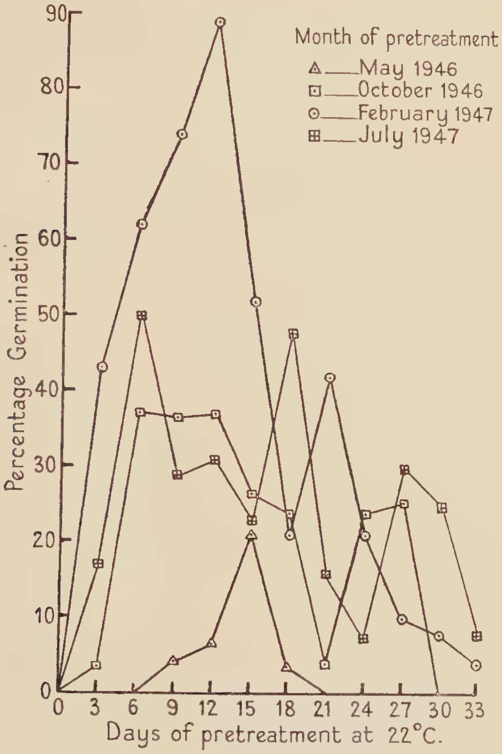

FIG. 2. The relationship between length of pretreatment at 22° C., the date of initiating pretreatment, and the germination of 1945 seeds.

From Fig. 2 it may be seen that whilst there were obvious differences in the curves of germination and in the maximum percentage of germination achieved in the four series, each was alike in showing a period of pretreatment to be necessary before the sample reached its maximum germination capacity. The data obtained from the experiments begun in April 1946, October 1946, and February 1947 might be taken as a clear indication that after-ripening does influence germination, for the peak germination percentage in each experiment was 21, 37, and 89 per cent. respectively. If this supposition is correct it would seem that after-ripening took place progressively during the time that the dry seed was stored in the laboratory.

The results of the germination study begun in July 1947 suggested that this explanation might not be valid, for as may be seen from Fig. 2, the germination curve showed two maxima and the peak germination was only 50 per cent. It seemed unlikely that the differences between the February curve and the July curve could be attributed to the loss of seed vitality, for a peak germination of 72 per cent. was obtained in further experiments on 1945 seed in November 1947. Thus the differences might have been due either to a further manifestation of after-ripening or, alternatively, to changed experimental conditions. Consideration of the data on the germination of 1945 seed alone might suggest that the variations described were due to some change in experimental conditions, particularly as reproducible results were only obtained when samples of seed were pretreated simultaneously. However, a comparative experiment on the effect of pretreatment on the germination of seeds harvested in three different years showed that after-ripening was probably the major factor responsible for the differences in the germination curves shown in Fig. 2.

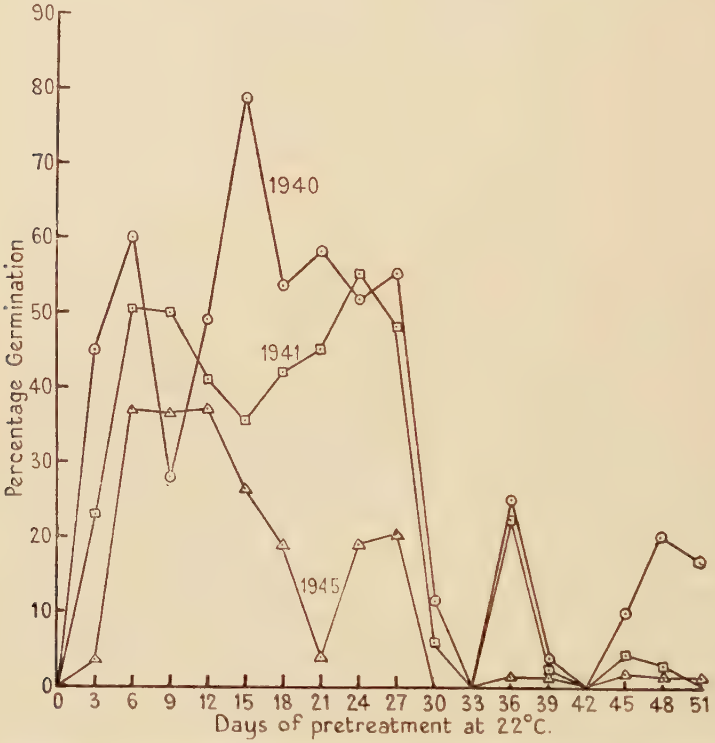

FIG. 3. The effect of length of pretreatment at 22° C. on the germination of seeds harvested in 1940, 1941, and 1945. (Pretreatment initiated October 1946: standard error of each mean germination percentage = ±3·6.)

Fig. 3 gives the data obtained by germinating samples of 1940, 1941, and 1945 seed at the same time by the standard method. This experiment was begun in October 1946 and the pretreatment of the three seed samples was initiated simultaneously. It will be observed from Fig. 3 that the germination capacity of the seeds of each sample increased during the first phase of pretreatment and that the respective peak germinations of the 1940, 1941, and 1945 seeds were 78, 58, and 37 per cent. The maximum germination percentages of the 1940 and 1941 seeds were higher than that of the 1945 seed in the replicate of the experiment illustrated by Fig. 3, but the results as a whole were significantly different from those shown in Fig. 3. However, additional experiments have indicated that the differences between the results shown in Fig. 3 and those of the replicate experiment could be attributed to the fact that in the replicate experiment the temperature exceeded 22° C. during the first 24 hours of pretreatment.

Now in these comparative experiments the seeds were all exposed to the same experimental conditions and consequently the differences between the peak germination capacities of the youngest and older seed samples cannot be ascribed to experimental error. Furthermore, the experiments illustrated in Fig. 2 show that in October 1946 at least 89 per cent. of the 1945 seeds were viable, whereas at the time of this experiment only 37 per cent. was induced to germinate together. In view of all these results it is considered that the germination of the seeds of *Striga hermonthica* is greatly influenced by after-ripening.

Certain additional experiments suggested that the rate of after-ripening is enhanced when dry seed is stored in the laboratory, as compared with the rate of the process in the native habitat. Thus when germination studies were carried out on 1940 and 1941 seeds in 1947, up to 42 per cent. of the seeds were induced to germinate in the absence of the stimulating solution. Furthermore, at this stage of after-ripening, the results of replicated experiments, separated by time-intervals of up to 7 days, did not differ significantly. The data obtained using 1940 seed in February 1947 are shown in Fig. 4; the data on the germination of 1941 seed in May 1947 are given in Fig. 5. As in previous experiments it will be seen that in the presence of the stimulating solution the amount of germination first increased as pretreatment was extended and then diminished after 27 days of pretreatment. It will be observed that in each figure the curve of germination, in the presence of the stimulating solution, is no longer cyclic and that the peak germinations of the 1940 and the 1941 seeds were 82 per cent.$\pm 3{\cdot}3$ and 63 per cent.$\pm 2{\cdot}3$ respectively. Both figures show a cyclic rise and fall in the percentage of spontaneous germination, but whereas with the 1940 seeds the maximum spontaneous germination was induced after 24 days of pretreatment; with the 1941 seeds this occurred after only 9 days of pretreatment. These differences were presumably due to the use of seed of different ages and not to the experimental technique. It must be emphasized, however, that, with one exception, spontaneous germination did not take place in the pretreatment vessels. It was induced only by incubating pretreated seeds, moistened with distilled water, for 48 hours at 32° C. In the exceptional case 5 per cent. of the seeds germinated spontaneously in the pretreatment vessels after 1940 seeds had been pretreated for 24 days at 22° C.

The effect of a second pretreatment on germination

It may be seen from Figs. 1 to 5 that after 15 days of pretreatment in the experiment illustrated in Fig. 1 and after 27 days in the other experiments the amount of germination diminished. In some experiments, such as those whose data are shown in Figs. 2 and 3, the percentage of germination at the end of the experimental period approached zero. Clearly it was desirable to ascertain whether pretreatment at 22° C., for the periods shown in Figs. 1 to 5, resulted in the death of the majority of the seeds, or alternatively whether the small amount of germination at the end of each experimental period was due to the seeds passing into a secondary state of dormancy towards the end of each experiment.

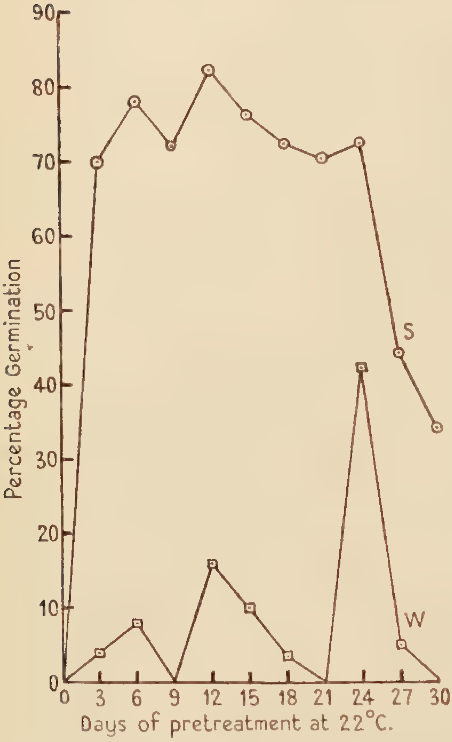

FIG. 4

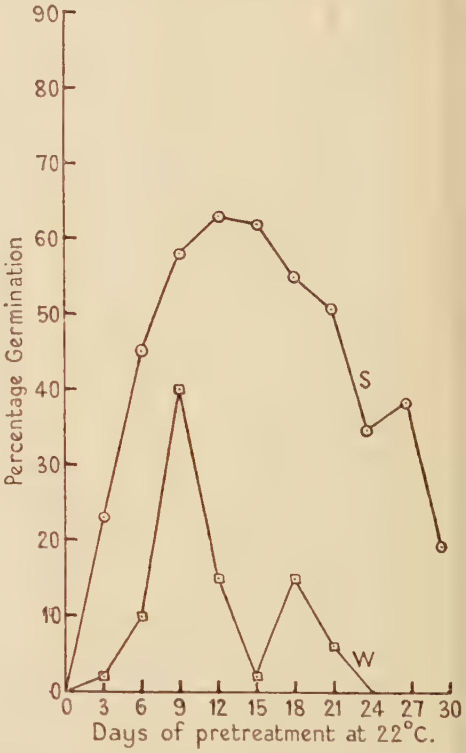

FIG. 5

FIG. 4. The effect of length of pretreatment at 22° C. on germination in the presence of the stimulating solution (s) and on germination in distilled water at 32° C. (w) 1940 seed. (Pretreatment initiated February 1947: standard error of each mean germination percentage = ±3·3.)

FIG. 5. The effect of length of pretreatment at 22° C. on germination in the presence of the stimulating solution (s) and on germination in distilled water at 32° C. (w). 1941 seed. (Pretreatment initiated May 1947: standard error of each mean germination percentage = ±2·3.)

Brown and Edwards (1944) showed in the case of the seeds of *Striga lutea* that if the temperature of pretreatment was increased at the time when the germination capacity of the seeds was falling, the germination capacity was enhanced compared with that at the initial temperature of pretreatment. This

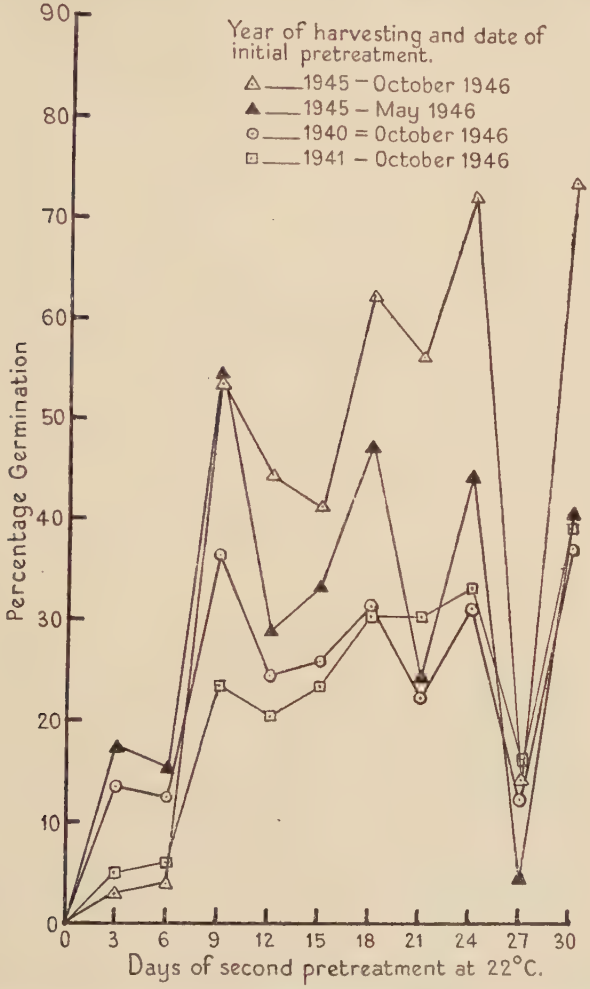

FIG. 6. The effect of length of a second pretreatment at 22° C. on the germination of seeds harvested in 1940, 1941, and 1945 respectively. For details see text. (Second pretreatment initiated in November 1946: standard error of each mean germination percentage = ±2·0.)

method was without effect on the germination of the seeds of *Striga hermonthica*. However, the loss of seed vitality during pretreatment in the experiments illustrated by Figs. 1 and 3 was not higher than 50 per cent. in the oldest seed and was much less than this in the 1945 seed. This was demonstrated by drying the seed at the end of each experiment and applying the germination technique to the dried seeds. Fig. 6 shows the relationship between the germination of seeds harvested in 1940, 1941, and 1945 and the length of the second pretreatment. Each point plotted in the figure is the mean percentage germination of six replicates. The data shown in Fig. 6 were obtained by drying the seeds remaining at the end of the experiments illustrated by Figs. 1 and 3 for 48 hours over concentrated sulphuric acid and then applying the germination technique to the dried seeds. The second pretreatments at 22° C. of the four seed samples were initiated simultaneously in November 1946.

It may be seen from Fig. 6 that the peak germination percentages of the 1945 seeds were higher than those in the initial experiments. Thus during the first pretreatment the peak germination was 21 per cent. when pretreatment was begun in April, and 37 per cent. when begun in October. The peak germinations obtained as a result of the second pretreatment were 52 and 74 per cent. respectively.

The differences in the maximum germination capacities of the samples of 1945 seed during the second pretreatment at 22° C. cannot be explained at this stage of the investigation. Nevertheless, it is clear that many of the seeds became dormant once more during pretreatment, in the experiments illustrated by Figs. 1 and 3.

An initial pretreatment at a temperature exceeding 22° C. was found to reduce markedly the capacity of the seeds to germinate after a second period of pretreatment at 22° C. Thus when seeds harvested in 1940 and 1945 were pretreated for 27 days at 27° C. and at 32° C., the maximum germination observed, after drying followed by pretreatment at 22° C. and exposure to the stimulating solution, was 10 per cent.

The relationship between temperature of pretreatment and germination

Various early experiments suggested that if seeds were pretreated at a higher temperature than 22° C. the effect of this would be not merely to shorten the period of pretreatment required by the seeds to enable them to reach their maximum germination capacity, compared with the period at 22° C., but to reduce their germination capacity. To determine the relationship between temperature of pretreatment and germination two comparative experiments were carried out simultaneously. In these experiments seeds harvested in 1940 and 1945 were pretreated at four different temperatures, namely 12° C., 22° C., 27° C., and 32° C. Two samples of each year's seed were pretreated at each temperature and three replicate lots of seed from each pretreatment vessel were exposed to the stimulating solution at times separated by 3-day intervals.

Fig. 7 shows the effect of pretreatment at the four different temperatures on the germination of seeds harvested in 1940. Pretreatment in this experiment was begun in November 1946. Statistical analysis of the data given in Fig. 7 showed that the differences in the germination percentages of the replicates and those between the germinations of the two seed samples were not significant.

In this series it will be seen that where seeds were first pretreated at 32° C. and 27° C. and then incubated for 48 hours in germination vessels containing the stimulating solution, the maximum amount of germination was less than that induced after a period of pretreatment at 22° C. The maximum percentage germinations were 22, 56, and 81 per cent. respectively. In addition to this it will be observed that the germination capacities of seeds pretreated at either 27° C. or 32° C. were never higher at any one stage than the comparable germination capacity of seed pretreated at 22° C. Furthermore, no more seeds germinated after 24 days of pretreatment at either 27° C. or 32° C. Temperatures of pretreatment exceeding 22° C. had a similar effect on the germination capacity of 1945 seed.

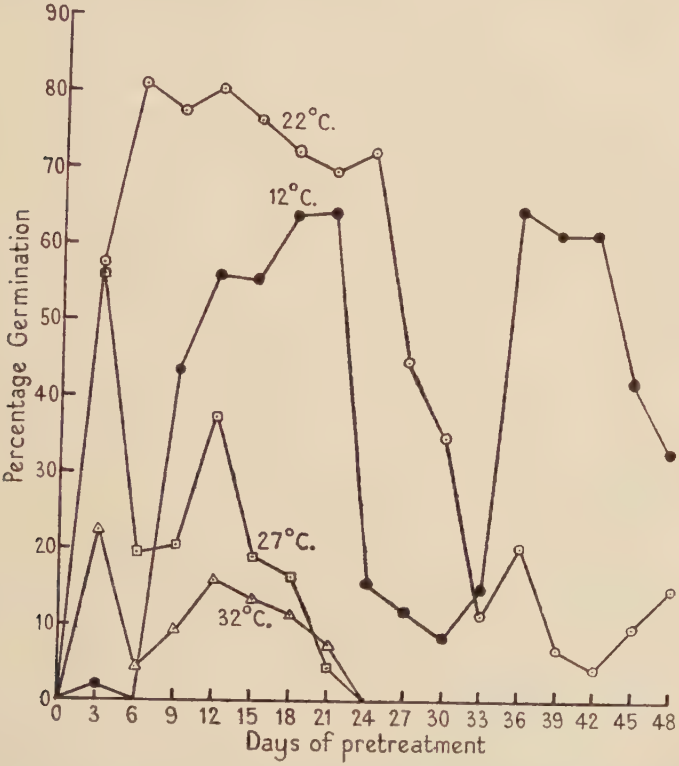

FIG. 7. The relationship between length of pretreatment, temperature of pretreatment (12° C., 22° C., 27° C., and 32° C.), and the germination of 1940 seed. (Pretreatment initiated November 1946: standard error of each mean germination percentage = ±3·8.)

Additional experiments on seeds harvested in 1940 and 1945 have shown that if seeds were pretreated at temperatures exceeding 22° C. for as little as 3 days, and were pretreated from then on at 22° C., the germination curve resembled that given after pretreatment for the whole period at the initial temperature. Thus whatever this temperature effect may be, it seems that the seeds do not recover from it during subsequent pretreatment.

The germination data obtained by exposing seeds pretreated at 12° C. to the stimulating solution shows certain features of considerable interest. It may be seen from Fig. 7 that during pretreatment at 12° C. the germination capacity first increased to a peak value of 65 per cent., diminished to less than 10 per cent., and then rose once more to 65 per cent. These data suggest that some of the seeds were in a state of secondary dormancy from the 21st to the 33rd days of pretreatment. Further pretreatment broke this dormancy so that after 36 days 65 per cent. of the seeds were able once more to germinate.

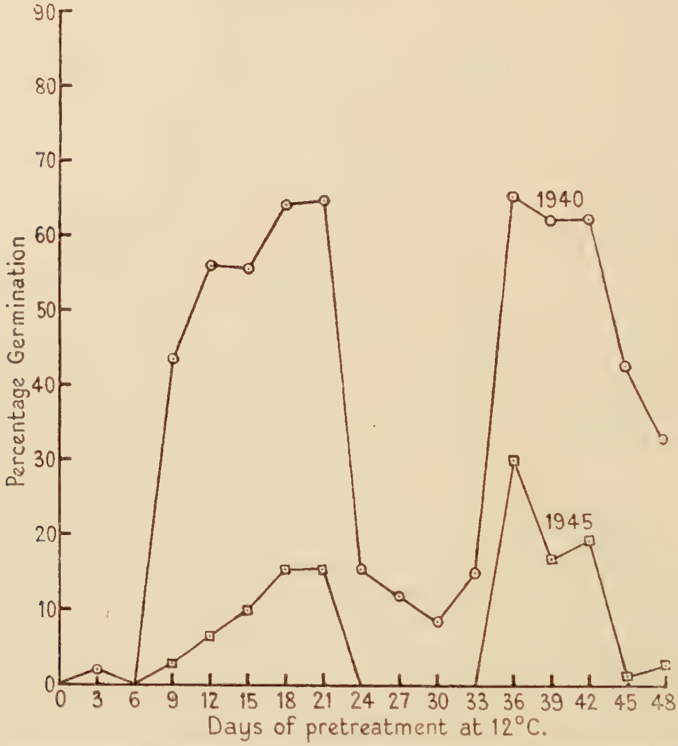

FIG. 8. The relationship between length of pretreatment at 12° C., the age of the seed (1940 and 1945), and the germination capacity. (Pretreatment initiated November 1946: standard error of each mean germination percentage = ±3·8.)

Essentially the same type of germination curve was obtained when 1945 seeds were pretreated at 12° C. Fig. 8 shows the data of the germination capacity of 1945 seed during pretreatment at 12° C. The comparable curve of 1940 seed, taken from Fig. 7, is included in Fig. 8. Subsequent experiments have shown that a curve of germination capacity with two maxima is always obtained when seeds are pretreated at temperatures from 12° C. to 20° C. and the germination data appropriately plotted. Thus whereas the characteristics of the various germination curves, drawn from the germination data of seeds pretreated at 22° C., have differed considerably, those curves derived by plotting the germination capacity during pretreatment at temperatures from 12° C. to 20° C. always showed certain similar features.

The effect on germination of diluting the standard stimulating solution

The relationship between germination and dilution of the stimulating solution was found to be influenced by two factors, namely, the age of the seed and the amount of pretreatment. This was shown by two experiments. In the first 1945 seed was pretreated at 22° C. from the end of May 1946, and the pretreated seed was incubated for 48 hours at 32° C. in germination vessels containing various dilutions of the stimulating solution. In the second experiment seeds harvested in 1940 were treated similarly. The pretreatment of this seed was begun in February 1947.

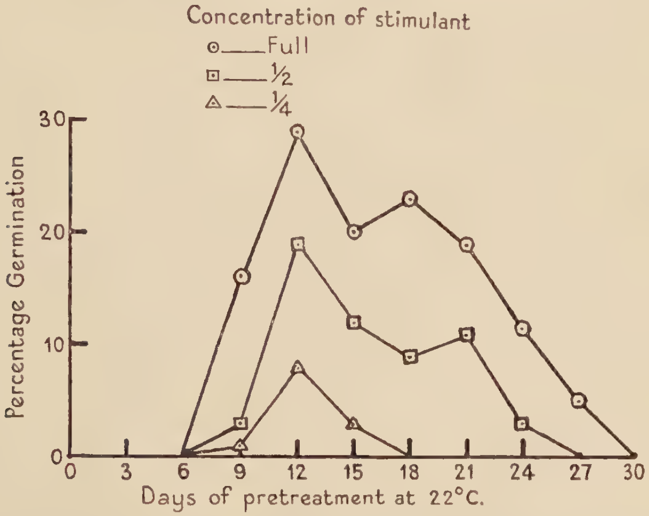

FIG. 9. The relationship between the length of pretreatment at 22° C., the concentration of the stimulating solution, and the germination of 1945 seed. (Pretreatment initiated May 1946: standard error of each mean germination percentage = ±1·8.)

Fig. 9 shows the effect of various dilutions of the stimulating solution on the germination of 1945 seeds after periods of up to 30 days of pretreatment. In this experiment glass-distilled water was added to the standard solution of the stimulant to give dilutions of 1:1 and 1:3. Two samples of seed were pretreated at 22° C., and at 3-day intervals seeds were withdrawn from the pretreatment vessels, and three replicate lots of seed from each sample were incubated for 48 hours in contact with each dilution of the stimulating solution. Each point plotted therefore represents the average value of six determinations of germination percentage. An Analysis of Variance showed the effect of stimulant-dilution to be highly significant, whereas the differences between seed samples and replicates were not significant.

It will be seen from Fig. 9 that between the 9th and 27th days of pretreatment the germination induced by treating the seed with the half-strength stimulating solution was lower than that resulting from the exposure of the seeds to the standard solution. Furthermore, the ratio between the germination induced by the standard solution and that by the half-strength solution was not constant during pretreatment. This ratio was 0·19, 0·65, 0·60, 0·39, 0·57, and 0·27 at the 9th, 12th, 15th, 18th, 21st, and 24th days of pretreatment respectively.

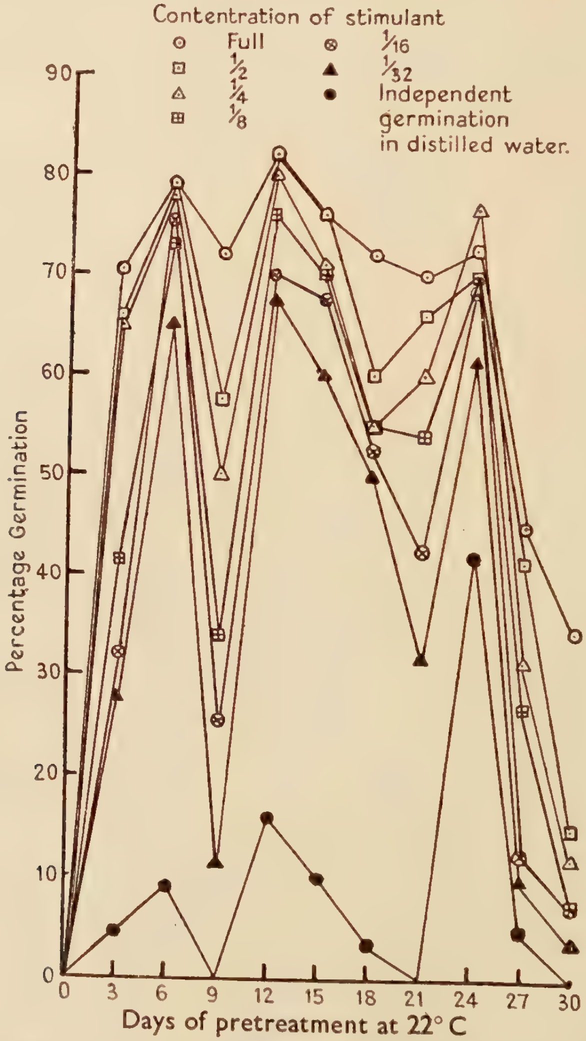

Fig. 10. The effect of dilution of the stimulating solution and length of pretreatment at 22° C. on the germination of seeds harvested in 1940. (Pretreatment initiated February 1947: standard error of each mean germination percentage = ±3·3.)

When 1940 seeds were used in a similar experiment begun in February 1947, the stimulating solution was further diluted. In all, dilutions of the stimulating solution of 1:1, 1:3, 1:7, 1:15, and 1:31 were employed, together with the standard solution and a control series with distilled water. The data of this experiment are given in Fig. 10. The Analysis of Variance of this data showed that the differences between the amounts of germination of the two samples of seed when exposed to either the standard solution or to distilled water were not significantly different. On the other hand, whereas the replicates did not differ significantly, the amounts of germination of seeds from the two samples after other treatments did differ significantly. However, as the dilution effects always showed a similar trend, the two sets of data were combined. Each point plotted in Fig. 10, therefore, is the mean germination percentage of two series each of three replicates.

The dilution effects shown in Fig. 10 are very striking and it will be immediately apparent that they are very different from those shown in Fig. 9. It may be seen from Fig. 10 that stimulant-dilution was least effective in reducing the germination at each of the peak phases of independent germination. At these times, namely the 6th, 12th, and 24th days of pretreatment, the germination was not reduced by dilutions above 1:15, 1:7, and 1:15 respectively. At other times, such as the 9th and 21st days, the effect of dilution on germination was considerable.

The nature of these effects is at present obscure. If, as suggested by Brown and Edwards (1946), in the case of *Striga lutea*, the seed manufactures the stimulant itself at the time of peak independent germination, it would mean that with *Striga hermonthica* the stimulant is progressively formed and destroyed as pretreatment proceeds. This may seem unlikely, but insufficient evidence is available at present either to confirm or refute this hypothesis.

DISCUSSION

It will be clear from the work described that a period of pretreatment followed by a relatively short period of exposure to a suitable stimulating solution is necessary before the optimum germination of the seeds of *Striga hermonthica* will take place. The two processes, though complementary, seem to be independent, for within the experimental limits the optimum temperature for pretreatment was 22° C., whilst that for treatment with the stimulating solution was 32° C.

The variation in the germination curves, obtained by plotting the germination capacities of the seeds in the presence of the stimulating solution during pretreatment at 22° C., could possibly be ascribed to one or more of three main factors. These are: (*a*) differences between samples of the experimental material due to the use of a small seed population, (*b*) variation in the conditions of pretreatment and in the conditions of the treatment with the stimulating solution, and (*c*) differences in the state of the dry seed immediately prior to the initiation of pretreatment.

It is thought that the seed population used in the pretreatment vessels was large enough to be fully representative, for 0·5 g. of *Striga hermonthica* seed contains at least 40,000 seeds, and hence this was neglected as a determining factor. The conditions of pretreatment and exposure to the stimulant also cannot be the major factor in determining the differences, for in the comparative experiments carried out on 1940, 1941, and 1945 seed the peak germination capacity of the older seed samples was always higher than that of the 1945 seed whether the pretreatment temperature was 12° C. or 22° C.

All things considered, it seems that the major factor responsible for the variation in the curves of germination frequency after pretreatment at 22° C. must be the condition of the seed immediately prior to the initiation of pretreatment, or in other words the stage of after-ripening. This after-ripening process must be of long duration when the seed is stored dry under tropical conditions, as comparatively high germination in the absence of the stimulating solution was only brought about in January 1947 in the case of 1940 seed and in May with 1941 seed, that is, after at least 6 years of storage. The changes which took place in the capacity for independent germination during the storage period in this laboratory would also indicate that after-ripening proceeds more readily at temperatures lower than those normally encountered in the native habitat.

It would be premature to attempt to interpret much of the experimental data presented at this stage of the investigation. However, the problems raised have suggested that a study of the drift of respiration, both during pretreatment and whilst the seed is in contact with the stimulating solution, would be of great value in elucidating some aspects of the germination process. It is hoped to publish an account of such work in the near future.

SUMMARY

1. The optimum germination of the seeds of *Striga hermonthica* will not be realized unless they are exposed to a suitable stimulating solution.

2. Data are presented which show that a period of exposure to moisture is necessary before the seeds are placed in contact with the stimulating solution if more than 0·1 per cent. are to germinate. Following the terminology of Brown and Edwards (1944) this moisture-treatment has been termed 'pretreatment'.

3. From germination curves, obtained by plotting germination percentage against length of pretreatment at 22° C., it has been shown that after-ripening has a considerable influence on the germination of the seeds of *Striga hermonthica*. Studies on seed of four different ages have shown that the peak germination capacity increases as the seed ages and that the shape of the curve of germination is influenced by the age of the seed.

4. The capacity of the seed to germinate in the absence of the stimulant was found to increase with age. When seeds harvested in 1940 and 1941 were pretreated in February and May 1947 respectively, 42 per cent. germinated in the absence of the stimulating solution.

5. When pretreatment at 22° C. was prolonged beyond a certain limit the germination capacity of the seeds approached zero. It is considered that this was due, except when the seeds were almost fully ripe, to the seeds passing into a condition of wet dormancy. It has been shown that this secondary dormancy could be overcome by drying the seed and applying the germination technique once more.

6. Pretreatment at temperatures of 27° C. and 32° C. reduced the peak germination capacity compared with that determined after pretreatment at 22° C.

7. The germination curve obtained by applying the standard germination technique to seed pretreated at 12° C. showed two maxima irrespective of seed age.

8. The relationship between length of pretreatment, germination percentage, and dilution of the stimulating solution was found to vary with the age of the seed. When 1945 seed was used 6 months after harvesting, it was found that dilution of the stimulating solution reduced the germination, compared with that induced by the standard solution. In experiments in which seed harvested in 1940 was employed it was found that, compared with the standard solution, a dilution of the stimulating solution of 1 : 31 reduced the germination. The effect of dilutions of 1 : 1, 1 : 3, 1 : 7, 1 : 15, and 1 : 31 was found to vary with the stage of pretreatment, but dilution had the least effect at the 6th, 12th, and 24th days of pretreatment. These times corresponded with those of the peak phases of independent germination.

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Observations on the Biology of *Fomes annosus*, with Particular Reference to East Anglian Pine Plantations

I. The Outbreaks of Disease and Ecological Status of the Fungus

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With Plates XII and XIII and one Figure in the Text

INTRODUCTION

A ROOT-DISEASE of pines (*Pinus sylvestris* L. and *P. nigra var. calabrica* Schneid.) has recently caused serious damage in some plantations established by the Forestry Commission in East Anglia. Macdonald (1939) first drew attention to these outbreaks, which were then confined to young trees planted on former woodland sites. From about 1941 onwards there was a more serious development of the disease, which killed considerable numbers of 20-year-old pines, chiefly on former arable land. Research into the biology of *Fomes annosus* Fr., which was apparently the causal fungus, was started in September 1945, at the suggestion of Professor F. T. Brooks, F.R.S. This paper gives a brief summary of the existing information about *F. annosus*, describes the characteristic features of the East Anglian outbreaks, and discusses the status and general biology of the fungus. An earlier paper (Rishbeth, 1949) is concerned with the implications of the disease for forestry practice.

SUMMARY OF EXISTING INFORMATION ABOUT *F. ANNOSUS*

Fomes annosus is recorded as a frequent cause of root and butt rot in conifers in the northern hemisphere. Serious outbreaks of disease have occurred, for instance, in India, Russia, Norway, Bosnia, France, and the U.S.A. Although the distinction is not always made in the literature, it appears that the killing of young conifers by the fungus is also fairly widespread. Thus Rennerfelt (1947) records that 20–30-year-old pine stands in south-west Sweden were badly attacked. Bornebusch and Holm (1934) refer to the death of young conifers, including pines, following the replanting of a former spruce plantation in Denmark. Serious losses of pines have been reported from France, particularly the central plateau and the Landes and Sologne districts, and also from Belgium and Germany. More recently, Wagener and Cave (1946) have described the killing of 200-year-old pines (chiefly *Pinus jeffreyi* and *P. ponderosa*) in a dry, sandy region of California. One of the few records of a similar nature from the British Isles is given by Anderson (1924), who described the

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dying of groups of pines in Northumberland and Lanarkshire. Although *F. annosus* is widespread as an agent of butt rot (Peace, 1938), it is apparent that the outbreak in East Anglia is the first of its kind on any considerable scale in this country.

Much cultural work has been carried out on *F. annosus*. Cartwright and Findlay (1934) give the growth rates on malt agar at various temperatures: the maximum is at about 23° C., when the daily increase in diameter of a Petri-dish culture is 13 mm. Weis and Neilsen (1927) found that the most favourable hydrogen-ion concentration for growth on malt agar lay between pH 4·0 and 4·5. Rennerfelt (1944) found that growth in a liquid medium containing purified salts and sugar was very poor in the absence of thiamin. Extracts of humus or compost gave marked increases in growth. *F. annosus* has been found to produce no less than sixteen enzymes (Miller, 1943). Since cellulose is decomposed in addition to lignin, a typical white rot is produced in wood. No worker has been able to obtain growth of the fungus on forest humus or soil unless this was previously heat-treated. Hiley (1919) obtained growth from mycelium and spores on sterilized larch twigs, blocks of larch wood, roots of larch and Weymouth pine, and various soils. He attributed the failure to grow on unsterilized soils to inhibition by bacteria. Treschow (1941) found that no growth was obtained on a soil which had been sterilized and then inoculated with *Trichoderma* sp., whereas vigorous growth followed on soil similarly inoculated with *Bacillus subtilis* or *Actinomyces* sp. Despite these findings, many workers, including Falck (1930) and Rennerfelt (1944), state that the fungus spreads from tree to tree in the humus layer.

Most previous investigations on the parasitic activities of *F. annosus* have been concerned with the problem of butt rot. There has been a considerable divergence of opinion on the mode of infection. Hartig (1894), who carried out the earliest important work on the fungus, stated that spores are brushed off on to roots by burrowing rodents, and that germination of the spores is followed by penetration of the cortex. Further infections were believed to take place by growth of the fungus from root to root where these are in contact or have fused. Huet (1936) made a similar diagnosis for severe outbreaks in Belgium. Lagerberg (1936), however, stated that infection of spruces can only take place through dead roots. Anderson (1924) thought it probable that roots are weakened by the diffusion of toxic substances from decaying stumps and that infection then follows. Růžička (1928) believed that invasion by *F. annosus* follows asphyxiation of the roots caused by excessive compactness of the soil, waterlogging, or a coarse humus covering on the surface. Rhoads and Wright (1946) thought it probable that entry occurs through wounds above ground level in *Tsuga heterophylla*.

In two instances recently the killing of pines has been specifically mentioned. Wagener and Cave (1946) found that, in California, *F. annosus* does not require wounds or dead tissues before the root is invaded. Day (1946, 1948) similarly concluded that, in East Anglian plantations, *F. annosus* can infect living tissues which have received no mechanical injury, but he suggested that invasion by the fungus is secondary to severe injury of the root system brought about by adverse physical conditions of drought or waterlogging.

It is hardly surprising that a wide variety of soil conditions has been associated with outbreaks of root and butt rot. Different conifers may vary greatly in their resistance to *F. annosus* on a given type of soil, as may a single species on a range of soil types. Resistance may also be affected by climate and other factors. Many workers agree, however, that the disease is likely to be severe in plantations established on former arable land. The prevalence of disease on old woodland sites is also frequently mentioned, but it has only once been clearly associated with the previous thinning of a plantation (Hepting and Downs, 1944). The conditions associated with the killing of trees by *F. annosus* have received little attention.

THE OUTBREAKS OF DISEASE

(i) *The affected areas*

The disease has developed in two main areas. The first, comprising the Thetford and Swaffham forests, lies for the most part in the area known as Breckland, that is, in north-west Suffolk and south-west Norfolk. Owing to the wide variety of diseased plantations and the comparative nearness to Cambridge, most of the work was done here. The second area, comprising the Rendlesham and Tunstall forests, lies a few miles from the coast of east Suffolk.

In both areas the rainfall is low, the average annual figure for the last 10 years being 21 in. The mean monthly temperatures for the same period range from 3° C. (January) to 17° C. (July). In Breckland the soil is derived from a chalky glacial drift and interglacial sands, the underlying formation being chalk. Large areas are covered by wind-blown sand. The soils are uniformly sandy, but show considerable variation chemically: where the chalk is near the surface the calcium carbonate content and pH value are high, but in other areas the base status and pH value are low and well-developed podzols may be present. The natural vegetation is largely grassland with societies of *Pteridium aquilinum* (L.) Kuhn and *Carex arenaria* L., but considerable stretches of heath are dominated by *Calluna vulgaris* (L.) Hull. Much derelict arable land is also present. In east Suffolk, on the other hand, the soil is derived from a sandy or gravelly drift beneath which is Norwich Crag. With the exception of land formerly cultivated, the soils are very acid. *Calluna vulgaris* is dominant over nearly the whole area, and grows more luxuriantly than in Breckland.

(ii) *The recent history of the areas*

Since the development of disease in the plantations is influenced by the former status of the sites, it will be convenient to refer briefly to the recent history of the affected areas. In the early part of the nineteenth century much of Breckland was cultivated for the first time. Associated with this, many windbreaks were established, the commonest type being a single line of *Pinus* *sylvestris*, but larger shelter strips comprising mixtures of conifers and deciduous trees were also planted. There were few large woods before 1840 and the trees in them were predominantly deciduous. Later in the century, however, several plantations were established in which the bulk of the trees were conifers. Large areas of grassland and heath still remained uncultivated, and in succeeding years much of the arable land became derelict. In the Rendlesham forest area only a few small woods and shelter belts existed before 1900.

From 1921 onwards the Forestry Commission established plantations on all types of land, the total area planted in Breckland being about 50,000 acres. Although records are incomplete, it is estimated that the areas planted on former woodland, heath (including grassland), and arable are in the approximate ratio of 1:3:1. The majority of former woodland sites were replanted from 2 to 4 years after felling, although occasionally a much longer period elapsed. Shelter strips adjacent to arable or heath sites were felled either before planting or at a variable time afterwards. In addition to planting, the Forestry Commission acquired a number of woods which had been established before 1922. *Pinus sylvestris* is the tree most commonly planted, and, after that, *P. nigra* var. *calabrica*. To a lesser extent *Pseudotsuga taxifolia* (Poir.) Britt., *Larix decidua* Mill., and a number of other species occur.

(iii) *The sources of infection*

Root disease has killed young trees on former woodland sites right from the beginning of replanting. Areas replanted after fire also show disease at an early stage. Deaths often occur 2 years after planting and continue for a variable period, sometimes as long as 19 years. In such plantations it is common to find groups of dead trees, around which each year one or two more lose vigour and die. By contrast, disease has appeared in plantations established on former arable or heath chiefly after thinning. In such sites there are also many instances of trees dying at the edge of felled woodland strips or along the border of 'racks' cut for access, or of 'rides' cut for fire protection. The most devastating attacks, however, have all followed thinning carried out in plantations about 15 years old. Much of this was of the 'pre-thinning' type, where relatively few coarsely growing trees were taken out, but there were also experimental thinnings, some of which were very heavy. As with plantations on former woodland, the trees characteristically die in groups, often circular, and this may lead to the formation of gaps up to 25 metres across.

In every site where the disease has developed, *Fomes annosus* is present in stumps. The source of infection is usually near the centre of gaps caused by the disease. Stumps in such places do not invariably contain *F. annosus*, but the likelihood of its former presence in them is suggested by the type of rot and by the fact that similar stumps in the area still contain the fungus. In every site containing infected stumps, moreover, it is possible to find living roots actively invaded by *F. annosus*.

In nearly half of the seventy sites investigated, infection has arisen from stumps of the previous crop. Of the remaining sites, where infection followed thinning in, or felling near, the standing crop, over a half were formerly arable. By contrast, only one-tenth of the remaining sites were formerly *Calluna* heath. This is partly because early thinning is infrequent in plantations on such sites and also because, when thinned, they suffer far less damage than similar plantations on former arable. Serious outbreaks of disease, such as those occurring on former arable, are comparatively recent because thinning was not carried out before 1937 and was widespread only from about 1942 onwards.

(iv) *The symptoms of disease*

The symptoms of disease and severity of attack are very similar in *Pinus sylvestris* and *P. nigra* var. *calabrica*. In the following account no distinction is made between the two species unless otherwise stated. In affected trees above 15 years of age the growth rate usually falls off for a year or two before death, and the crown shows various abnormalities: only one year's growth of needles is borne, or the needles themselves are unusually short. By the time these symptoms appear the root system is extensively invaded by the fungus. Resin may exude copiously from the roots, which often have characteristic paper-like sheets of mycelium between the bark-scales (Pl. XII, A). Before the crown dies, sporophores of *F. annosus* may appear at the base of the trunk in sheltered situations. The bark-beetle *Myelophilus piniperda* L. frequently invades the trunk either after death or when the root system is heavily infected; in the latter condition, symptoms of disease may still be lacking in the crown. This invasion may well hasten the death of the tree and create the opportunity for wood-rotting fungi such as *Polystictus abietinus* (Dicks.) Fr. and *Pleurotus mitis* (Pers.) Berk. to enter. Sporophores of these fungi were occasionally present some way up the trunk while the crown was alive. The early invasion by bark-beetles helps to explain the very rapid rotting of the trunks of trees killed by this root disease; in the absence of sporophores of *F. annosus*, the deaths might easily be attributed to bark-beetles, as has happened in the U.S.A. (Wagener and Cave, 1946).

(v) *The isolation of* F. annosus

Evidence that *Fomes annosus* is the cause of this type of attack was chiefly derived from a detailed examination of root systems. Chips of bark and wood from diseased roots were dipped momentarily in alcohol, surface-sterilized by immersion for 5 minutes in a solution of calcium hypochlorite (about 2 per cent. free chlorine), and then plated on malt agar. Cultures were incubated for 10 days at 22·5° C. When it was required merely to demonstrate the presence or absence of *F. annosus* in wood samples these were cleaned, wrapped in newspaper, thoroughly wetted with tap-water, and kept in glass containers at room temperature (17°–20° C.). Under such conditions the presence of *F. annosus* was indicated by the production of conidia, usually after 4–5 days, but only after 8–10 days when the tissues were resinous. These conidia are borne as characteristic heads (Pl. XIII, A and B), as originally described by Brefeld (1889), and are easily recognizable under the low power of the microscope. Since the conidia cannot be confused with those of any other common wood-inhabiting fungus, this direct incubation of wood or bark gives reliable results, and far fewer isolations on laboratory media are required. The proportion of positive results obtained by direct incubation is often greater than that obtained by plating, partly because *F. annosus* is somewhat irregularly distributed in the tissues, but also because saprophytic fungi grow freely on malt agar and often obliterate the slower growing parasite. The method was also used for large diseased roots. These were cut at selected places to expose the bark and wood, divided into suitable lengths, and then split longitudinally. After incubation, conidia indicated the approximate distribution of *F. annosus* in the bark and wood.

Trees recently affected by root disease almost invariably contained *F. annosus* in some part of the root system. The only exception was with trees affected by *Armilaria mellea* (Vahl.) Fr., which, however, was confined to sites previously bearing hardwoods or a mixed crop. The species of *Phytophthora* isolated from the diseased trees by Day (1946) was not found, nor were any other fungi of pathogenic significance. *F. annosus* was isolated in pure culture from the wood of pine roots which had recently been killed, although the bark sometimes had a superficial growth of other fungi, such as *Trichoderma viride* Pers. ex Fr., *Cylindrocarpon radicicola* Wollenw., and *Pencillium* spp. Such fungi also commonly occurred on the surface of healthy roots. In affected roots the boundary between healthy and diseased tissues, frequently marked by resin exudation, regularly contained *F. annosus*. The same fungus was also isolated from roots in the earliest stages of disease, when the affected portions were only a few centimetres in length. These observations strongly suggest that *F. annosus* was the causal parasite.

(vi) *The mode of infection*

Investigation of early stages of infection showed that, in every instance, the fungus had invaded a living root at a point where it touched another already infected. The first trees to be killed are always infected from stump roots (Pl. XIII, C), but such trees may in turn transmit the disease to adjacent ones. Infection occurs either on one or more healthy lateral roots at some distance from the bole, or at the bole itself. Stump roots never infect living roots unless they are in intimate contact with them. This, however, is not an important limitation to the spread of disease, since root contacts are frequent in plantations from about the eighth year onwards, and actual root fusions are moderately common. Later stages of infection are more difficult to interpret, but the source of infection can usually be found. The observation that initial attack by *F. annosus* is confined to the root system agrees with most previous work, but the part played by root contact in the spread of infection has not always been clearly stated.

THE ECOLOGICAL STATUS OF *F. ANNOSUS*

(i) *Habitat and host-range*

In East Anglia *Fomes annosus* is one of the commonest wood-rotting fungi present in coniferous stumps. During the investigation it was found in stumps of *Pinus sylvestris* L., *P. nigra* var. *calabrica* Schneid., *Pseudotsuga taxifolia* (Poir.) Britt., *Larix decidua* Mill., and *Picea abies* (L.) Karst. Parasitic attacks on all these species were seen and also on young *Chamaecyparis Lawsoniana* Parlatore. *F. annosus* was frequently present in stumps of *Betula pendula* Roth., but only occasionally in those of *Quercus robur* L. *Betula* showed a remarkable ability to survive attacks by the fungus: stumps with a considerable amount of rot often produced healthy shoots, and trees with a high proportion of rotted roots appeared normally healthy, though occasionally suffering wind-throw. *F. annosus* was isolated from the dying roots of 30-year-old *Quercus robur* L., *Fagus sylvatica* L., and *Populus italica* Moench., which were growing in a former shelter strip and were surrounded by numerous infected pine stumps. Since nearby trees, already dead, also contained *F. annosus*, it is probable that the fungus can kill these hardwoods under certain conditions. *Fagus*, without doubt, is killed sporadically soon after planting in some areas where infected stumps are present. Parasitic attacks on *Crataegus monogyna* Jacq. were also seen.

Sporophores of *F. annosus* were sometimes found on the stumps of *Ulex europaeus* L. cut out during clearing, and in one instance young pines near by had been infected from this source. The last and most surprising record is of *F. annosus* on *Pteridium aquilinum* (L.) Kuhn, on which it has been found four times. In one instance a rhizome had been infected by contact with a pine root, and the fungus was growing in the stelar tissues. In another a small sporophore was borne on the stump of a rotted frond. Tests made in the laboratory suggest that the fungus is unlikely to grow vigorously enough on bracken rhizomes to be a serious source of infection to trees. Attacks by *F. annosus* on the two hosts just mentioned have not previously been recorded, as far as is known.

In addition to these records from roots or stumps, *F. annosus* was occasionally found in sawn pine logs lying on the ground. A small amount of free mycelium was seen twice on litter and once on the floor of a vole tunnel, in each instance immediately beneath a sporophore of the fungus. Similarly, the only soil sample (out of about 250 tested) which produced colonies of *F. annosus* after plating a suspension of it in Dox's agar was obtained from beneath a sporophore. In other situations visible mycelium on the soil or litter always belonged to other fungi. No rhizomorphs of *F. annosus*, similar to those described by Miller (1943), were seen during the investigation; rhizomorphs present on the surface of roots rotted by *F. annosus* were found to belong to other basidiomycetes.

It is well known that the fungus may remain for years as an agent of decay in the stumps of trees which it has killed, but the means by which it colonizes the stumps of freshly felled trees has not been elucidated. Most workers have assumed either that it is already present in a dormant condition in the root system or that it enters from the soil or litter when the resistance of the tree is lowered by felling. The observations so far given throw little light on the problem, for it is notoriously difficult to isolate basidiomycetes from soil or litter by plating, and negative results are therefore not necessarily significant. Although, as described above, free mycelium of *F. annosus* was seen on litter, its distribution was so limited that little significance can be attributed to the observation.

(ii) *The growth of* F. annosus *on natural substrates*

If *F. annosus* can live saprophytically in soil or decomposing litter as well as in stumps, the potential source of infection to living trees is very great and, in some circumstances, invasion through mycorrhizal roots might take place. In view of the conflicting opinions about this in the literature, further evidence was sought.

In one experiment the ability of *F. annosus* to grow on freshly gathered samples of soil and litter from Breckland plantations was determined. To prepare the inocula, pine roots were cut into 5-cm. lengths, sterilized by autoclaving, and infected with agar inocula of the fungus. The roots, incubated at 22·5° C. in wide-necked stoppered bottles, were ready to use after 3 to 4 weeks. Sterile Petri dishes were half-filled with the soil to be tested, the soil surface was levelled, and a single inoculum of the type described above placed in the centre. If the soil was dry, it was brought to a moisture content of about 10 per cent. by the addition of sterile distilled water. The Petri dishes were placed in containers, to limit evaporation, and were incubated at 22·5° C. for 14 days. When the soil samples were previously sterilized by autoclaving, abundant growth from the inoculum always occurred but, with unsterilized samples, results were variable. With the latter the growth of mycelium from the inoculum was measured; the results, which are given in Table I, are grouped for convenience.

TABLE I

The Growth of F. annosus *on Unsterilized Soil and Litter from Breckland Plantations*

Description of sample.	Depth of sampling. (cm.).	Total no. of samples.	No. of samples giving growth (mm.) from the inoculum:			
			0.	1–2.	3–5.	>6.
Litter	0–1	16	14	2	0	0
Humus	1	17	17	0	0	0
Acid soil	8	13	6	4	1	2
" "	15–30	18	6	4	1	7
Alkaline soil	8	18	7	1	6	4
" "	15–30	20	5	1	3	11

It is clear that, under these conditions, growth of *F. annosus* in the humus layer or litter is either very restricted or does not occur. In the great majority of instances the inoculum was overgrown by *Trichoderma viride*, which suggests that the failure of *F. annosus* to grow may be associated with a type of antagonism. *F. annosus* in fact shows little or no capacity to invade litter in the plantations, for the fungus was never isolated from small branches, twigs, or needles which had been embedded in sporophores for some time. This debris normally contains species of *Penicillium*, *Trichoderma viride*, and other fungi.

F. annosus often grew better on unsterilized soil from a depth of 8 or 15–30 cm. than on humus or litter. The acid soils had a smaller proportion of samples supporting a moderate growth of *F. annosus* (groups 3–5 mm. and > 6 mm.) than had the alkaline ones, but the distinction between the two types of soil was not marked in this particular test. The growth of *F. annosus* on unsterilized soils has not been recorded before, possibly because weaker inocula and an insufficient variety of soil samples were used. In this experiment, however, growth on unsterilized soil was never profuse. Since much of the work carried out on root-disease fungi has shown that these must have a substantial 'base' before infection takes place (Garrett, 1944), it seems unlikely that *F. annosus* could ever achieve infection from a limited growth in the soil.

Field observations are of interest in this connexion. Mycelium of *F. annosus* was never seen growing into the soil even from heavily infected roots. The white pustules often present on the surface of such roots contained viable mycelium only when they were freshly formed; older pustules were discoloured yellow and contained other fungi. This suggests that other fungi prevent *F. annosus* from growing into soil. Another significant fact is that *F. annosus* was found in the root systems of only a small proportion (less than 4 per cent.) of pines killed by suppression. If the fungus were widely present in the soil or litter, it might be expected to invade a greater proportion of such trees, whose resistance just before death is presumably low. Infection experiments, to be described in a later paper, show that *F. annosus* does not necessarily enter even these few weakened trees from the soil, for such trees can be infected through stem wounds, which are often present, by means of airborne spores.

During this investigation *F. annosus* was never found on the root systems of trees planted on former arable or heath sites unless stumps were present in the immediate vicinity. The examination of root systems of pines blown over by a severe gale in March 1947 showed this relationship clearly. In two areas where thinning had previously been carried out, 16 out of 23 such trees had *F. annosus*, mainly in the lateral roots. In comparable sites only a short distance away, where there had been no previous thinning, the fungus was not found in the roots of 30 blown trees. Since roots are known to die during the normal growth of pines (Büsgen and Münch, 1929), opportunities for colonization by a fungus such as *F. annosus* would be likely to occur if it grew freely in the soil. The above evidence suggests strongly that mycelial growth does not occur widely in the soil, but is limited to root systems in certain situations. This does not rule out the possibility that spores of *F. annosus* are washed into the soil and remain viable for a short period, as is suggested by experiments to be described in a later paper. It is probably true to say, however, that *F. annosus* is not present to an extent capable of causing infection in plantations on former heath or arable land until stumps are produced by thinning or other operations.

In another experiment suspensions of basidiospores, obtained by washing off fresh spore deposits on glass slides with sterile distilled water, were sprayed on to the surface of moist soil samples in Petri dishes, which were then incubated at 22·5° C. for 10 days. Mycelium of *F. annosus* never appeared unless the soil was previously sterilized by autoclaving. It appears unlikely, therefore, that the fungus can colonize soil under natural conditions by means of basidiospores.

The ability of *F. annosus* to grow on pine roots was also tested. Roots were washed to remove soil, but the bark-scales were left intact as far as possible. Two 5-cm. lengths were placed parallel and a small distance apart in a sterile Petri dish moist chamber, and a pine-root inoculum of the type already described laid on them across the centre at right angles. Dishes were incubated in containers for 14 days at 22·5° C., and the surface growth of mycelium from the inoculum recorded. With root lengths previously sterilized by autoclaving, growth from the inoculum was always abundant. With similar unsterilized root lengths, growth was variable and was classified in groups as follows: 0, none; 1, slight; 2, moderate; 3, abundant. In one experiment roots taken from a depth of 20 cm. in the soil of two plantations, one of which was formerly chalk grassland (site A) and the other *Calluna* heath (site B), were tested in this manner. Since earlier experiments had suggested that the surface growth of *F. annosus* on roots from site B was likely to be poor, a test was made at the same time with roots from site A which had previously been encased by bark-scales taken from roots of site B. The results obtained from these three series of root samples, each ten in number, are illustrated in Pl. XII, B, and recorded in Table II.

TABLE II

The Surface Growth of F. annosus *on Unsterilized Pine Roots*

Type of root sample.	No. of samples giving a growth from the inoculum in the groups:			
	0.	1.	2.	3.
From former chalk grassland (site A) . .	0	0	0	10
,, ,, *Calluna* heath (site B) . . .	5	3	2	0
Composite: site A roots encased by site B bark-scales	2	6	2	0

There is a striking difference in the growth of *F. annosus* on roots from sites A and B. Since it can be shown that the growth rate of the fungus in the wood of such root samples is unrelated to the type of site from which the roots are taken, it is probable that purely surface differences were responsible for the result. This is supported by the fact that the failure of *F. annosus* to grow out from the inoculum on to roots of site B was often associated with overgrowth by *Trichoderma viride*. Furthermore, growth of *F. annosus* on roots from site A plus bark-scales from site B was almost as restricted as it was on intact roots from site B, which strongly suggests that the bark-scales of roots from this site contained fungi antagonistic to *F. annosus*.

These findings correspond closely to the state of affairs under natural conditions: a superficial mycelium of *F. annosus* is often present on diseased pine roots in the alkaline soils of former arable and chalk grassland, but very seldom on similar roots in acid soils of former woodland or *Calluna* heath. The ability of *F. annosus* to grow relatively freely on roots in alkaline soils is almost certainly associated with a deficiency of competing fungi, such as *Trichoderma viride*, on the root surface.

Basidiospores of *F. annosus* germinated and produced mycelium readily on the surface of sterilized roots. Mycelium was seldom produced on unsterilized roots under similar conditions, but the chance of establishment was increased by cutting to expose the bark and wood. Establishment of the fungus on living roots by means of basidiospores is therefore theoretically possible under natural conditions, especially where wounding occurs. Basidiospores produced mycelium readily on freshly sawn sections of pine stem, unless there was heavy contamination with other fungi. The possibility of stump infection by this means cannot therefore be disregarded.

(iii) *The interaction of* F. annosus *and* Trichoderma viride *on laboratory media*

Trichoderma viride commonly overgrows *F. annosus* on agar, soil, and wood in the laboratory at 22·5° C. In addition, it often replaces *F. annosus* in stumps under natural conditions. For these reasons, and because *T. viride* was already known to affect the parasitism of *Rhizoctonia solani* and other fungi, the interaction of the fungus with *F. annosus* was studied. Ten representative strains of *T. viride*, isolated from East Anglian soils or from pine roots, were used for this work. Observations were also made on two strains, producing gliotoxin and viridin respectively, kindly supplied by Dr. P. W. Brian.

In one experiment the interaction on malt agar at 22·5° C. was investigated. A colony of *F. annosus* was allowed to develop from an agar inoculum on one side of a Petri dish for 5 or 6 days before an inoculum of the faster growing *T. viride* was placed on the opposite side. The hyphae of *F. annosus* were always checked when they came in contact with those of *T. viride*, and some strains of the latter were similarly checked at first. However, all ten East Anglian strains of *T. viride* and the two provided by Dr. Brian eventually grew through the *F. annosus* colony, although their growth sometimes continued by submerged hyphae only. When this growth of *T. viride* was complete, attempts to reisolate *F. annosus* failed and microscopic examination showed that most if not all of the hyphae were dead. Colonies of *F. annosus* kept under similar conditions but in pure culture retained viability throughout the period of the experiment.

Although *F. annosus* grows most rapidly at about 22·5° C., it must rarely encounter temperatures as high as this under natural conditions. The average soil temperature over a period of 4 years at a station in Breckland, for instance, varied between 3·6° C. (January) and 16·9° C. (August) at a depth of 30 cm. It was thought important, therefore, to discover whether the interaction with *T. viride* was similar at lower temperatures. As a preliminary, their relative growth rates on malt agar were measured. The ten strains of *T. viride* already mentioned and ten of *F. annosus*, all but one from East Anglia, were used for this comparison. Growth rates were measured for two Petri-dish cultures of each strain at 10, 5, and 0° C., each colony being measured along two diameters at right angles. The figures in Table III are the average colony increases in radius per day in mm.

TABLE III

Growth rates of F. annosus *and* T. viride *on malt agar*

	10° C.			5° C.			0° C.		
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.
F. annosus	2·7	2·1	2·5	1·7	1·1	1·4	0·6	0·3	0·4
T. viride	6·4	1·7	4·5	3·7	0·3	2·0	0·1	0·0	0·0

At 10° C. *T. viride* grew faster than *F. annosus*, at 5° C. the two fungi grew at approximately the same rate, while at 0° C. *T. viride* hardly grew at all. Three strains of *T. viride* giving rapid overgrowth of *F. annosus* at 22·5° C. were then tested for their interaction with this fungus at lower temperatures. The malt-agar cultures were incubated at 22·5° C. until the fungus colonies had almost met, and were then transferred to 10, 5, and 0° C. The results are given in Table IV.

TABLE IV

The Interaction of F. annosus *and* T. viride *on malt agar*

+ denotes overgrowth by *T. viride*.
— denotes no overgrowth by *T. viride*.

Temp. (° C.).	Incubation period (months).	Strains of *T. viride*		
		1.	2.	3.
10	3	+	+	+
5	4	+	—	—
0	6	—	—	—

In one instance at 5° C. and in one at 0° C. *F. annosus* had started to overgrow *T. viride* very slowly. There is thus a tendency for the action to be reversed at low temperatures. This result has a bearing on some of the earlier observations. It is interesting, for instance, to find that the records of free *F. annosus* mycelium beneath sporophores were all obtained in late October or November, when the air temperature was below 10° C. The growth rate of *F. annosus* on unsterilized natural substrates is so slow below 10° C., however, that extensive colonization of soil or litter is unlikely to occur under these conditions.

It was possible to study the action of *T. viride* more closely by growing the two fungi on a thin malt-agar film mounted on a coverslip in a van Tieghem cell. Under these conditions some strains of *T. viride* had an effect on the *F. annosus* hyphae well before contact occurred. The *F. annosus* hyphae were wider than usual and produced numerous short branches almost at right angles; later they became extensively vacuolated and often burst, liberating a yellowish globule of disorganized protoplasm (Pl. XII, C and D). The effect was similar to that described by Weindling (1932) for *Rhizoctonia solani* but, although the *T. viride* hyphae lay very close to those of *F. annosus*, they did not enter them.

In view of recent work by Brian and Hemming (1945) and by Brian et al. (1946) on the production of antibiotics by *T. viride*, the activity of sterile extracts prepared from the ten strains was tested. The strains were grown in Weindling's medium (adjusted to pH 3·5) for 4 days at 25° C., and the cultures were then Seitz-filtered. The method of assay closely followed that described by Brian and Hemming (1945), except that conidia of *F. annosus* were used instead of those of *Botrytis allii*. Successive twofold dilutions of the extract to be tested were made with half-strength Weindling medium. Spore suspensions were added and mixed, and three drops were withdrawn from each on to a sterile microscope slide. The slides were placed in a moist atmosphere and incubated at 22·5° C. for 48 hours, when they were examined by microscope. The activity of the extract was measured by the highest dilution inhibiting germination of conidia to less than 1 per cent. The results are given in Table V.

TABLE V

The Activity of Seitz-filtered Extracts of T. viride *Strains, as shown by their Effect on the Germination of* F. annosus *Conidia*

Maximum dilution of extract inhibiting germination	No. of strains of *T. viride.*
0 (i.e. no dilution)	7 (East Anglian)
$\frac{1}{4}$	2 (,,)
$\frac{1}{2}$	1 (,,)
$\frac{1}{64}$	2 (Dr. Brian)

Germination was not inhibited in the controls.

When dilutions of gliotoxin were tested with separate suspensions of conidia and basidiospores of *F. annosus*, germination was inhibited up to the same dilution with each. Since Brian and Hemming (1945), working with *Botrytis allii*, record an inhibition of germination at far higher dilutions than those given above for conidia of *F. annosus*, the latter are probably less sensitive to toxins of *T. viride* than conidia of *B. allii*. Nevertheless, a marked difference in activity between the East Anglian strains of *T. viride* and those from Dr. Brian is evident. With none of these strains did a relation appear to exist between the ability to overgrow *F. annosus* on malt agar and the production of toxin, as judged by this method. *T. viride* can undoubtedly kill aerial hyphae of *F. annosus*, as happens on the glass surface above agar cultures in Petri dishes, and it is therefore likely that, when hyphae of the two fungi are in intimate contact, the local production of toxin is greater than the tests indicate.

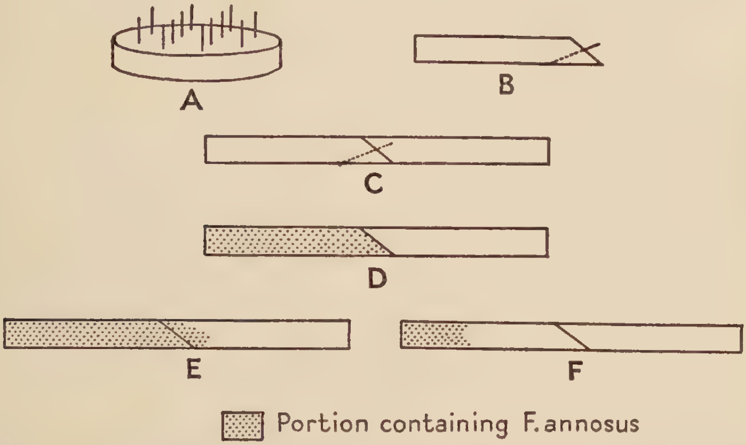
It may be concluded from these experiments that many strains of *T. viride* are antagonistic to *F. annosus*, although the effect is less marked below 10° C.

(iv) *The interaction of* F. annosus *with other fungi in pine roots*

Since there is extensive competition between fungi in stumps and dead roots, it is interesting to investigate the interaction of *F. annosus* with other fungi in woody tissues. A technique was therefore evolved by which fungi could be grown in pine roots. In order to obtain reproducible results it was necessary to obtain uniform growth of the fungi throughout the root samples and to use the roots soon after such growth was complete. For this, wood inocula were preferred to agar cultures. Inocula were prepared from blocks of pine wood about 5 cm. in diameter and 0·7 cm. thick. Twelve small pins were pushed through each block, which was then moistened and sterilized by autoclaving. The pine blocks (Text-fig. 1, A) were transferred to agar cultures of the fungi and incubated at 22·5° C. until they were well permeated by mycelium.

Meanwhile, freshly collected pine roots of diameter 0·5–1·0 cm. were cut into 5-cm. lengths, washed, and stripped of loose superficial bark. Each length was cut squarely at one end and obliquely at the other. A pin was fixed in each so that it projected through the obliquely cut end (Text-fig. 1, B). No pins, however, were inserted into root lengths to be inoculated with *F. annosus*. The roots were sterilized by exposure to the vapour of propylene oxide in stoppered bottles for 3 days, the same period being allowed for the dispersal of the vapour through the slightly raised stoppers. The root lengths were then firmly attached to the pine block inocula by fixing the squarely cut ends on to the projecting pins. Each inoculum, with twelve lengths of pine root attached, was incubated at 22·5° C. in a moist, sterile, wide-necked bottle. The root lengths were ready to use shortly after hyphae appeared on the obliquely cut ends.

In order to study interaction, root lengths containing *F. annosus*, as described above, were attached to roots containing other fungi so that the obliquely cut surfaces were adjacent (Text-fig. 1, C and D). Each pair, kept in position by the pin fixed earlier, was placed in a moist sterile test-tube. These tubes were incubated in covered containers either at 5° C. or at 15° C. For comparison, pairs were also made up in which root lengths artificially infected by *F. annosus* were replaced by lengths of naturally infected, rotted root. After a suitable interval (see Table VI) the root lengths were removed, split longitudinally, and reincubated for a short time until the relative positions of *F. annosus* and the other fungi in the wood could be observed. To prevent any great change in the relative positions of the fungi, this reincubation was carried out at 5° C. Advance (+) or replacement (—) of *F. annosus* was graded 1, 2, or 3; 3 indicating that either process was nearly complete. 0 indicated that there was no advance or replacement. Two possible results of interaction are illustrated in Text-fig. 1, E and F. The results of interaction between *F. annosus* and some other fungi are given in Table VI. Each figure of advance or replacement is an average obtained by assessing the interaction in three pairs of root lengths incubated under the given conditions. The fungi were isolated from pine roots or stumps in East Anglian plantations, with the exception of *Trichoderma viride*, strain 5, which was isolated from Breckland soil, and *Ceratostomella pini*, which was obtained from the Forest Products Research Laboratory, Princes Risborough. The basidiomycetes *Peniophora gigantea* and *Stereum sanguinolentum* were particularly abundant in pine stumps.



TEXT-FIG. 1. Diagrams illustrating a technique for studying interaction in pine roots (× ½). A, wood block of the type used as inoculum; B, root length with pin inserted; C, a pair of root lengths in position; D, the position of *F. annosus* before incubation; E, one possible result of interaction, scoring +1; F, another possible result, scoring —2.

In artificially infected root lengths at 5° C., *F. annosus* was replaced to some extent by two strains of *Trichoderma viride*, by *Torula ligniperda*, and *Peniophora gigantea*. The replacement of *T. viride*, strains 1 and 5, by *F. annosus* at this temperature parallels the reversal of interaction already described for agar cultures (Table IV). At 15° C. all four strains of *T. viride* replaced *F. annosus* rapidly, as did *Torula ligniperda* and *Peniophora gigantea*. On the other hand, *F. annosus* replaced the two species of *Ceratostomella* to some extent at both temperatures. 'Blue-stain' fungi similar to these sometimes invade pine roots soon after felling, and the result just given agrees with the observation that *F. annosus* often replaces them as it grows out from stumps

Table VI

The Interaction, as measured by Advance (+) *or Replacement* (−), *of* F. annosus *with Other Fungi in Lengths of Pine Root*

	Root lengths containing *F. annosus*			
	Artificially infected Incubated		Naturally rotted Incubated	
Root lengths containing the fungi.	at 5° C. for 6 weeks.	at 15° C. for 2 weeks.	at 5° C. for 6 weeks.	at 15° C. for 2 weeks.
Trichoderma viride Pers. ex Fr., str. 1	+1	−3	−2	−3
,, ,, ,, ,, 2	−1	−3	−3	−3
,, ,, ,, ,, 4	−2	−3	−3	−3
,, ,, ,, ,, 5	+2	−2	−3	−3
Torula ligniperda (Willkom) Sacc.	−2	−3	−3	−3
Ceratostomella pini Münch	+3	+2	0	0
C. pilifera (Fr.) Winter	+1	+1	−2	−3
Peniophora gigantea (Fr.) Massee	−1	−2	−3	−3
Polystictus abietinus (Dicks.) Fr.	+1	+1	−2	−2
Stereum sanguinolentum (A. & S.) Fr.	0	0	−2	−2

along the lateral roots. Stages in the replacement of *F. annosus* by *Peniophora gigantea* in stumps have often been seen. The results obtained for the other basidiomycetes suggest that there is little or no interaction with *F. annosus*, a conclusion which is supported by the observation that the parasite often exists in stumps with these fungi for a considerable period. In the naturally rotted root lengths *F. annosus* was presumably at some disadvantage with the other fungi, which could draw on the more substantial food-base of the fresh, artificially infected lengths. However, the very free growth of these other fungi into the rotted lengths suggests that *F. annosus* is readily replaced in rotted wood by many other fungi. The extent of replacement did not differ appreciably during the respective incubation periods at 5° C. and 15° C.

That *F. annosus* is often unable to compete with other fungi in dead roots is suggested by an experiment in which root lengths artificially infected with *F. annosus* were paired in the manner already described with samples of dead roots taken from pines killed by suppression. A great variety of fungi, including basidiomycetes, are found in such roots. For comparison, similar root lengths containing *F. annosus* were paired with root samples taken from living pines adjacent to the dead suppressed ones. After 2 weeks at 15° C. the extent to which *F. annosus* had advanced in these root samples was determined and graded as before. The results are given in Table VII. There was thus little or no growth in root samples from the dead suppressed trees but vigorous growth in those from the living trees.

The status of *F. annosus* is now clearer. It characteristically inhabits stumps and roots, in which it may remain for many years actively rotting the wood, and seldom if ever grows in soil or decomposing litter. By means of spores or mycelium it can colonize freshly cut pieces of root or stem provided that these

TABLE VII

The Growth of F. annosus *in Root Samples obtained from Dead (Suppressed) and Living Pines*

Root samples.		No. giving growth of *F. annosus* in the groups.			
Origin.	No.	0.	1.	2.	3.
Dead trees	18	13	5	0	0
Living trees	11	0	0	1	10

are not too heavily contaminated by other fungi. *F. annosus* is readily replaced in wood by various strains of *Trichoderma viride* and by *Peniophora gigantea.* Although it can invade wood already occupied by certain other fungi, its powers of invasion are limited; in this respect it resembles *Armillaria mellea* (Leach, 1939). For this reason it is probable that *F. annosus* infects trees through dead roots only rarely, if at all. This is contrary to the opinion of some earlier workers such as Lagerberg (1936), who were concerned with butt rot. In East Anglia pine roots appear to be normally healthy before invasion by *F. annosus*, a condition also found in similar outbreaks of disease in California (Wagener and Cave, 1946). In the plantations investigated stumps are the primary sources of infection, *F. annosus* passing from stump roots into living roots which touch them. As has been stated elsewhere (Rishbeth, 1949), stumps are infected in the first instance by air-borne spores of *F. annosus.* The observations and experiments which demonstrated this will be described more fully in a forthcoming paper.

SUMMARY

Fomes annosus has recently caused serious damage in some East Anglian plantations of *Pinus sylvestris* and *P. nigra* var. *calabrica.*

A summary of existing information about *F. annosus* is given. Most previous investigations on the parasitic activities of the fungus have been concerned with butt rot; in the present work, however, attention has been directed to the type of root disease associated with the killing of pines.

The areas affected have a low rainfall and sandy soils, which show considerable variation chemically. Trees are often killed on former woodland sites soon after replanting, but serious disease appears on former arable or heath sites only after thinning of the plantations. Affected trees regularly have *F. annosus* in some part of the root system.

The fungus is present in stumps in every site where the disease has developed. Infection takes place by the contact of living roots with ones already infected. The first trees to be killed are infected from stump roots, but such trees may transmit the disease to adjacent ones. This leads to the formation of considerable gaps, which are often circular.

Field observations suggest that *F. annosus* does not grow freely in the soil, but is confined to woody tissues. Growth of *F. annosus* on samples of

unsterilized litter or humus was either very restricted or did not occur. Limited growth was obtained on soil samples from depths of 8 and 15–30 cm. The fungus grew well on the surface of unsterilized pine roots taken from a plantation on former chalk grassland, but poorly on roots taken from a plantation on former *Calluna* heath. This difference was probably due to the abundance of competing fungi, such as *Trichoderma viride*, in the latter site.

An account is given of the interaction in culture of *F. annosus* with strains of *T. viride*, many of which are antagonistic to the former.

A technique is described by which the interaction of fungi in roots was studied. Although *F. annosus* can invade roots already occupied by certain fungi, notably some causing 'blue-stain', its powers of invasion are limited. It is rapidly replaced in roots and stumps by *Trichoderma viride* and *Peniophora gigantea*.

The writer is indebted to Professor F. T. Brooks, F.R.S., who gave valuable help and encouragement during the investigation, and Dr. S. D. Garrett for discussion and suggestions during its later stages. Thanks are due to many officers of the Forestry Commission for their assistance. Grateful acknowledgement is also made to the following: the Director of the Forest Products Research Laboratory, Princes Risborough, for identifying cultures of basidiomycetes and for supplying some cultures of 'blue-stain' fungi; Mr. E. W. Mason, of the Commonwealth Mycological Institute, for identifying certain fungus cultures; Dr. P. W. Brian for providing two strains of *Trichoderma viride*; and Messrs. F. T. N. Elborn and P. M. B. Walker for taking the photographs.

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EXPLANATION OF PLATES

PLATE XII

A. A pine root recently invaded by *F. annosus*, showing white mycelium where the superficial bark has been removed (↓) and resin exudation (right), which has caused soil to adhere. (×¼.)

B. The growth of *F. annosus* on unsterilized pine roots. In some instances white mycelium of *F. annosus* can be seen growing out from the inoculum, which is laid over two roots. (×¼.) Upper row: root samples from plantation on former chalk grassland (A), supporting good superficial growth of *F. annosus*. Middle row: root samples from plantation on former *Calluna* heath (B), supporting poor growth. Spore clusters of *Trichoderma viride* can be seen on the filter paper. Lower row: root samples from site A encased with bark scales from those of site B, also supporting poor growth.

C. Photomicrograph of normal hyphae of *F. annosus* growing in a thin film of malt agar. (×200.)

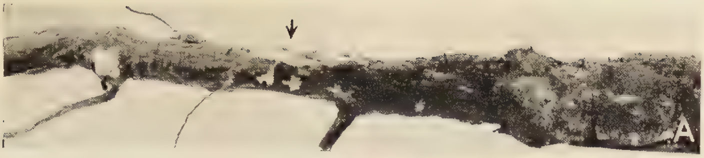
D. Photomicrograph of similar hyphae affected by toxin diffusing from a colony of *Trichoderma viride* about 2 mm. away. (×200.)

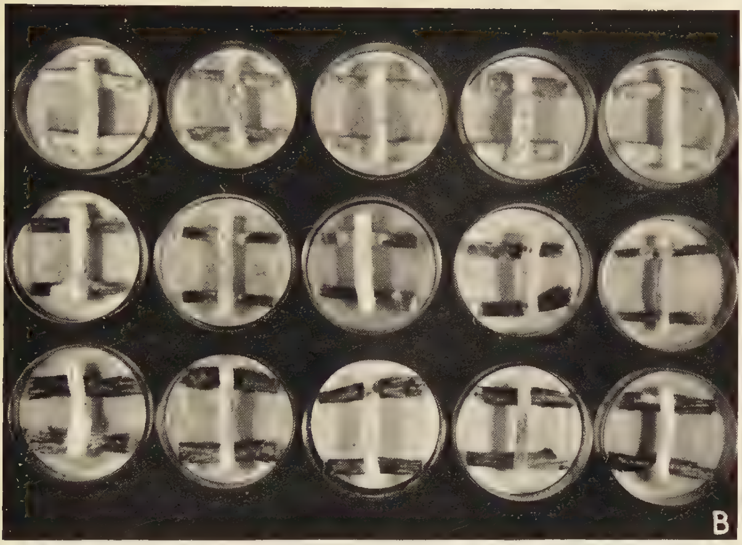
PLATE XIII

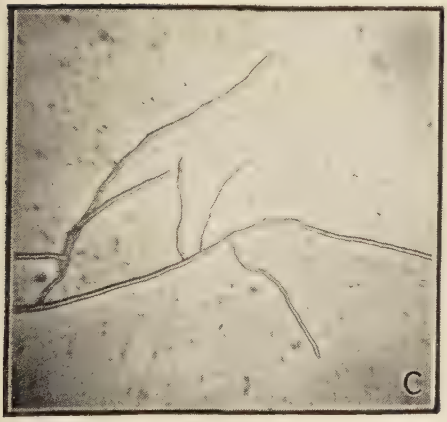
A. Photomicrograph of conidial heads of *F. annosus* formed during the incubation of a piece of infected root in the laboratory; side view. (×80.)

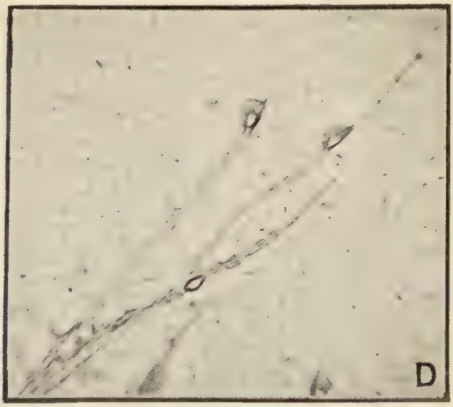
B. Photomicrograph of conidial heads of *F. annosus* produced under conditions similar to the last; surface view, showing their characteristically dense formation. (×60.)

C. A small Scots pine (*Pinus sylvestris* L.) killed by *F. annosus*, showing its roots in contact with infected stump roots (↓).









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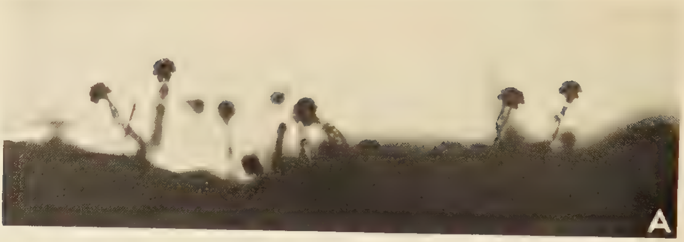

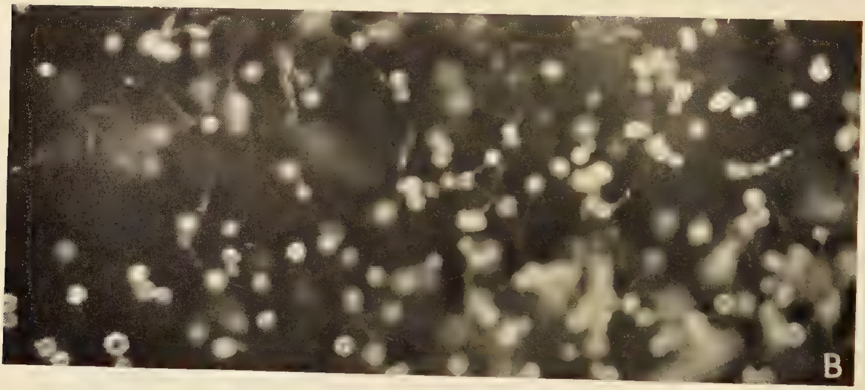

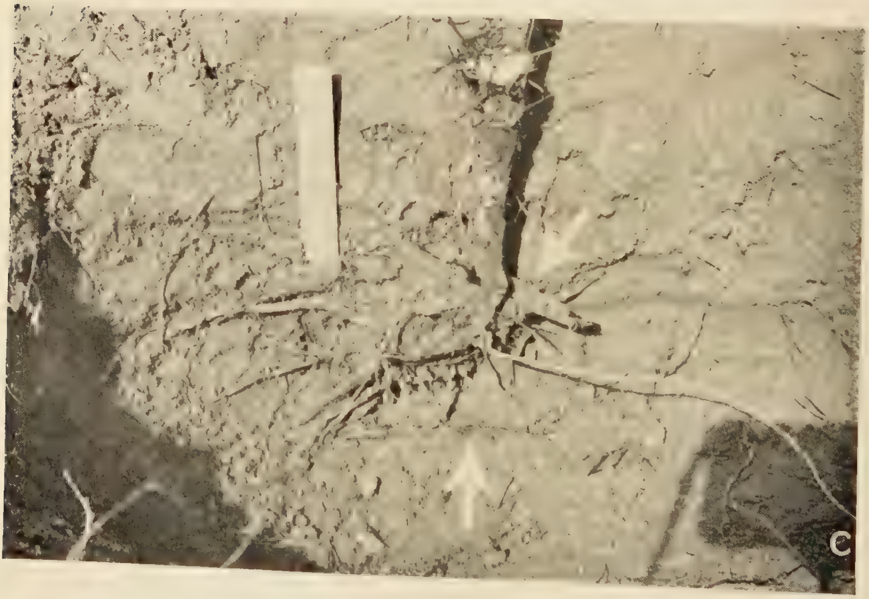

J. RISHBETH

The Relation between Cell Dimensions, Osmotic Pressure and Turgor Pressure

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With one Figure in the Text

IT appears always to have been assumed from the earliest expositions of Ursprung and Blum (1916–24) and Thoday (1918) onwards that on the intake of water by a plant cell the turgor pressure increases in linear relation to the cell volume. Diagrams after Thoday (1918) based on this assumption are repeatedly reproduced: the idea is implicit in many accepted calculations. Relative volumes of cells in different conditions of turgor have moreover been estimated by cubing the ratios of over-all cell lengths or diameters and such estimated volumes have been assumed to be indicative of the relative turgor pressures and inversely of the osmotic pressures in the different states. Inasmuch as calculations and arguments are frequently based upon these assumptions, Lyon (1942), for instance, having based upon them an argument to prove the existence of a non-osmotic force in the water relations of potatoes, it appeared the suppositions were worthy of closer scrutiny. On examination they both turn out to be incorrect. This results from failure to recognize two important principles, which, if taken into account, profoundly modify the conclusions to be reached from arguments in which calculated turgor and osmotic pressures are involved. The calculated plasmolytic-cryoscopic discrepancies of Lyon (1942), for instance, readily fall to less than half their values when the principles to be put forward below are taken into account and may even disappear altogether. The data therefore take on a very different complexion when presented as evidence for a non-osmotic force in the case under investigation.

The principles which have escaped attention are (*a*) that the osmotic pressure should (neglecting small corrections for changes in degree of dissociation, hydration, imbibition, &c.) be taken as inversely proportional not to the volume of the whole cell or block of tissue (as by Lyon, 1942) but to the volume of the vacuole only, and (*b*) that there can be no linear relation between the turgor pressure and any cell dimension or the cell area or volume: its relation to the linear dimensions must, if the cell-wall material obeys Hooke's Law, be hyperbolic. Some cells, though we do not yet know how general this phenomenon may be, evidently have anomalous elastic properties and do not obey Hooke's Law (cf. data by Stow, &c., quoted by Tamiya, 1938, and Thoday, 1949), the modulus increasing with the extension and the stretching therefore

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being relatively greater at first and falling off. In such cases a linear relation is conceivable as a resultant of two superimposed effects but remains unlikely. The principle to be discussed may operate together with other principles and its effect may or may not be offset by them, but that it must always operate is mechanically inevitable.

The terms concerned will be used as follows:

Suction pressure = diffusion-pressure deficit (Meyer, 1938; Currier, 1944) = water-absorbing power (Thoday, 1918) = 'Suction force' = *Saugkraft* (Ursprung, 1916, &c.) = osmotic pressure of bathing solution with which the cell would be in equilibrium on the assumption that only purely osmotic forces are concerned = osmotic pressure—turgor pressure.

Turgor pressure = outwardly directed pressure on the cell wall (tending to burst the cell) in dynes per sq. cm. of area of inner surface of cell wall on which it acts.

Wall pressure = the inwardly directed reaction to turgor pressure = the pressure in dynes per sq. cm. of area of the protoplast, exerted on the cell contents by reason of the tension in the stretched wall. Wall pressure is (*at equilibrium*) necessarily equal and opposite to turgor pressure.

All the above terms are as used and defined by Molz (1926).

Wall tension = force per unit transverse sectional area of cell wall in dynes per sq. cm. of wall material. It results from stretching of the wall by turgor pressure and is decided in magnitude by the amount of stretching and the modulus of elasticity of the wall material. Young's Modulus for the wall substance would be given by

$$Y = \frac{\text{wall tension}}{\text{relative extension}},$$

whence wall tension = $Y \times \frac{l}{L}$, l being the increment in length of an original length L, and l/L the relative extension of the wall. It may be noted that l/L is equal to the relative change in any linear dimension of the cell.

In pursuance of criticism (*a*) above, it has to be remembered that not all the constituent phases of a block of tissue or even of a single cell are susceptible to changes in volume in response to changes in the osmotic properties of the bathing solution. The vacuole is the part of the cell most generally recognized as undergoing such volume changes. On the view that the inner protoplasmic membranes are principally responsible for osmotic swelling and plasmolytic phenomena (cf. Höffler, 1940), the vacuole might indeed be the only constituent of the cell to exhibit osmotically induced volume changes, and in this case the whole of the change in volume of a block would be due to that of the vacuoles only. It is true that if the outer membranes also possess any appreciable degree of semi-permeability to the solution concerned, the protoplast will also be expected to contribute some measure of osmotic volume changes and that it might in any case undergo some change in volume through changes in imbibition. Imbibitional changes can, however, only be relatively small and osmotic volume changes of the protoplast, if the latter be supposed to maintain a volume in equilibrium with the osmotic concentrations of the vacuole and bathing solution, can only be brought about in so far as the outer membrane is semipermeable, and its degree of functional semipermeability (cf. Höffler, 1940) is probably relatively slight. The chances are then that the protoplast, which may represent as much as 30 per cent. of the volume of the cell (Currier, 1944), will not participate markedly in volume changes. In any case, whatever the position with respect to the protoplast (which like the other points at issue is amenable to experimental investigation shortly to be undertaken), it is certain that there are other constituents of a block of tissue which cannot actively participate in volume changes. The cell walls, the intercellular spaces, and all casual constituents of the cells such as oil drops or starch grains must all be reckoned as almost completely static in this respect. In calculating the osmotic pressure of cell sap in any one osmotic state from the osmotic pressure in another state and a volume ratio, it is obviously the ratio for the vacuoles only which should be taken into account and not the volume ratio for the whole cell or block of tissue in the different states.

The extent of osmotic dilution of the cell sap by absorbed water, always assumed in the literature to be related to cell volume by a straight line, thus depends upon the change in volume of the water phase of the vacuole alone and does not therefore necessarily bear any simple relation to the volume of the whole cell or whole block of tissue. The aggregate volume of all the 'static' constituents not contributing to volume changes should be deducted from the total volume of tissue and only the remainder taken to represent the extensible vacuole: changes in volume should be reckoned on this remainder and not on the total volume. On this basis a given change in volume will of course appear as a larger percentage change in vacuole volume, and a change in osmotic pressure may appear considerably larger than when calculated on the basis of the volume changes of the whole cell or block. Where the vacuoles are relatively small or there is a large volume of cell inclusions such as starch, the percentage changes may easily appear at least three times as great on this basis. Since it is argued that the vacuoles may normally be the only important contributors to osmotically induced changes in volume, it is evident that errors arising from basing calculations on the volume changes of whole blocks of tissue or even of single cells may be very considerable.

Criticism (*b*) is less simple. It is generally realized in connexions of diverse kinds from problems in surface tension and the design of boilers to the strain on heart muscle with increased ventricular volume that the larger a container becomes the greater is the stress necessary in its walls to resist a given internal pressure, and the greater is the strain produced in the walls by any given pressure. Conversely, the smaller the container the greater is the pressure set up inside by a given strain in the walls. The application of this well-known principle to cells expanded by turgor pressure, however, appears to have completely escaped attention. That it cannot be justifiably ignored on the grounds of the small range of variation of cell size in most cases can be immediately shown by quantitative calculations as well as by the simple consideration that many cells expand by as much as 30 per cent. or more of their flaccid dimensions, and that the range of turgor pressures involved is from zero to the maximum decided by the osmotic pressure of the cell contents. In these circumstances it cannot be assumed that the results on the assumption of an arithmetic relationship will be practically the same as those on a hyperbolic basis. It is also incorrect to suggest that any reasonably satisfactory approximation may be achieved by taking the turgor pressure as proportional to the length or area of the cell instead of the volume, since, as will be shown, it may become more nearly proportional to all these functions in turn. The best approximate law therefore depends upon the range of values as well as on the Young's Modulus for the wall substance, so that the most suitable approximation to apply cannot be predicted.

The true relation between turgor pressure and cell dimensions for a cell with an isotropic wall obeying Hooke's Law will be realized from the following considerations.

Consider a partially turgid spherical cell.

Let $R =$ the mean radius (i.e. the radius measured to the centre of the cell wall = mean of inside and outside radii) at incipient plasmolysis,
$r =$ the increment in mean radius due to turgor pressure, and
$d =$ the thickness of the cell wall in flaccid condition.

Then the cross-sectional area of wall material at incipient plasmolysis

$$= 2\pi Rd,$$

the mean area of cell wall (= mean of inside and outside surface areas)

$$= 4\pi R^2,$$

and the volume of wall material = mean area × thickness

$$= 4\pi R^2 d.$$

When a cell stretches, the mean area of wall increases in proportion to $(R+r)^2$. If the volume of wall material be assumed to remain constant and stretching only to alter its shape,

$$4\pi R^2 d = \text{const.} = k \text{ (say)}.$$

Hence d varies inversely as $(R+r)^2$ or

$$d = k/(R+r)^2.$$

The cross-sectional area of wall in any state

$$= 2\pi(R+r)d = 2\pi(R+r)k/(R+r)^2 = 2\pi k/(R+r).$$

Let $F =$ the total force between the two imaginary hemispheres of the cell, due to turgor pressure and equal and opposite to that due to wall pressure, let $W =$ the wall tension, or force per unit area of cross-section of wall, and let $Y =$ Young's Modulus for the wall material.

Then $$Y = W \times \frac{R}{r} = \frac{F}{\text{wall area}} \times \frac{R}{r},$$

or $$Y = \frac{F}{2\pi k/(R+r)} \times \frac{R}{r} = \frac{FR(R+r)}{2\pi kr}$$

and $$F = 2\pi krY/R(R+r).$$

But Turgor pressure, T, $=$ force per unit area on median plane of cell

$$= F/\pi\left(R+r-\frac{d}{2}\right)^2,$$

or, neglecting the half wall-thickness as small compared with R,

$$T = F/\pi(R+r)^2$$

$$= \frac{2\pi krY}{\pi R(R+r)^3} = \frac{2krY}{R(R+r)^3}$$

$$= K'r/(R+r)^3, \quad \text{where } K' = 2kY/R$$

$$= K'r/(R^3+3R^2r+3Rr^2+r^3),$$

or, neglecting powers of r which are small compared with R,

$$T = K'r/R^2(R+3r) = Kr/(R+3r),$$

where $K = K'/R^2$.

Hence Turgor pressure $= K\dfrac{r}{R+3r}$.

A plot from this equation gives a rectangular hyperbola with an intercept R (the radius at incipient plasmolysis) at $T = 0$. The form of the graph is shown by the heavy curve in Fig. 1.

The steepness of the curve obviously depends partly upon K, which

$$= K'/R^2 = 2kY/R^3, \qquad \text{since } K' = 2kY/R \text{ (above)}$$

$$= 2d(R+r)^2Y/R^3, \qquad \text{since } k = d(R+r)^2.$$

The turgor pressure, therefore, though in any case bearing a hyperbolic relation to a linear dimension of the cell, increases more rapidly with linear extension the thicker the cell walls (d), the greater the elasticity modulus (Y), and the smaller the initial size of the cell.

Conversely, considering extension as a result of turgor pressure, it is evident that the linear dimensions of a cell, always bearing a hyperbolic relation to the turgor pressure, increase the more rapidly with turgor the thinner the cell walls, the smaller the elasticity modulus, and the larger the cell.

The relation may be made clearer by substituting simple hypothetical values in the equation, letting K and the initial radius $=$ unity, and plotting the turgor pressure and relative cell radii, areas, and volumes for extensions of 10, 20, 30, &c., up to 100 per cent. The values obtained from the equation $T = r/(1+3r)$ are given in Table I and plotted in Fig. 1. The range plotted

TABLE I

r.	$R+r$.	W ($\propto r$).	Area $\propto(R+r)^2$.	Volume $\propto(R+r)^3$.	$R+3r$.	Turgor pressure (relative values $= 100r/(R+3r)$).
0·0	1·0	0	1·0	1·0	1·0	0·0
0·1	1·1	1	1·21	1·32	1·3	7·7
0·2	1·2	2	1·44	1·73	1·6	12·5
0·3	1·3	3	1·69	2·20	1·9	15·8
0·4	1·4	4	1·96	2·75	2·2	18·2
0·5	1·5	5	2·25	3·38	2·5	20·0
0·6	1·6	6	2·56	4·1	2·8	21·41
0·7	1·7	7	2·89	4·92	3·1	22·6
0·8	1·8	8	3·25	5·84	3·4	23·52
0·9	1·9	9	3·7	6·7	3·7	24·4
1·0	2·0	10	4·0	8·0	4·0	25·0

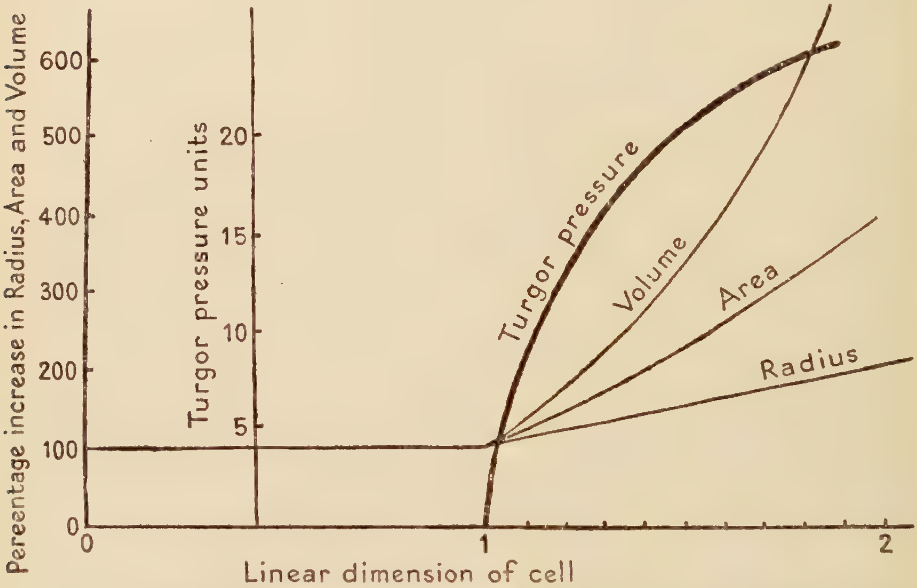

FIG. 1. Plots of the hypothetical figures given in Table I. A linear dimension, e.g. the diameter, of a cell is supposed to be unity in the condition of incipient plasmolysis. The thin curves represent respectively the percentage increases in radius, area, and volume as the cell expands, all plotted on the same scale. The thick curve represents the corresponding increase in turgor pressure in the arbitrary units of Table I, and is a plot of the equation $T = KR/(R+3r)$, being therefore a rectangular hyperbola. For further explanation see text.

(up to 100 per cent. increase in diameter) is more than that of which most cells are capable. The wider range makes the principles and differences between the graphs more obvious, but the differences over normal experimental ranges are sufficient to lead to serious errors if calculations are made on the assumption of proportionality to cell volume.

It may be seen from the graph (Fig. 1) that, although the steepness of the curves naturally depends upon the units and scales chosen, with increase of a linear dimension of the cell the corresponding turgor pressure rises most rapidly at first and later less rapidly. Of the curves to cell dimensions that for volume naturally rises most steeply, that for area less so, and that for radius least. The turgor pressure, therefore, at very small cell extensions runs most nearly parallel with the volume of the cell, at greater extensions it is most closely parallel with the area, and for greater extensions still with the radius, there being, however, no good approximation to proportionality to any of these functions. The slopes of the curves and therefore the ranges over which these generalizations apply moreover depend upon the modulus of elasticity, the thickness of the cell walls, and the actual size of the cell, so that no generalization can be safely applied. Any sort of extrapolation or interpolation may be wildly incorrect unless a full calculation be made taking the hyperbolic relation to true vacuole volume into account.

It may be noted, though it may be of less practical application than the general relation, that the full equation for the turgor pressure will be

$$T = \frac{2rd(R+r)^2Y}{R^3(R+3r)}.$$

It is now possible to examine the effect of applying these principles to some existing calculations and conclusions. Those of Lyon (1942) will serve well as an example.

Lyon (1942) determined the suction pressures and lengths of blocks of potato tuber in the flaccid, normal, and fully turgid states. From the ratios of the lengths he calculated the relative volumes by cubing. From the ratios of these volumes he obtained the theoretical values for the osmotic pressure of the original (normal) material. He then calculated the osmotic pressure at saturation by multiplying that at incipient plasmolysis by the inverted volume ratio and took this value as equal to the turgor pressure at saturation. The original turgor pressure was calculated from this by multiplying by the ratio of the difference between the volumes of normal and plasmolysed blocks to the difference between the volumes of saturated and plasmolysed blocks. (This, of course, is assuming a linear relation between turgor pressure and block volume.) The original suction pressure was then calculated from the difference between these calculated values for the original osmotic pressure and turgor pressure. Lack of agreement with the experimentally observed value of the suction pressure of the original material was taken to show the inadequacy of the purely osmotic view and that a non-osmotic force was concerned.

It is instructive to examine one of Lyon's calculations and to make a recalculation from his data, taking into account that the turgor pressure bears a hyperbolic, not linear, relation to the extension, and that the ratios of volumes of vacuoles in the different states should be used instead of the ratios of the volumes of the whole blocks.

Lyon's own data and calculation will be summarized first. Altering the format of presentation slightly to facilitate comparison with the new working they run as shown in Table II.

TABLE II

Data and Calculation after Lyon with Lay-out adapted for Comparison with Table III

		At incipient plasmolysis.	In original condition.	At saturation.
Measured suction pressures	=	21·5	8·7	0·0 atm.
Measured lengths of blocks	=	0·595	0·619	0·658 cm.
Calculated relative volumes	=	2,106	2,380	$2{,}860 \times 10^{-4}$ cm.^{3}
Osmotic pressure at incipient plasmolysis = suction pressure at incipient plasmolysis				
∴ Calculated original osmotic pressure $= 21{\cdot}5 \times \frac{2{,}106}{2{,}380}$	=		19·05 atm.	
Calculated osmotic pressure at saturation $= 21{\cdot}5 \times \frac{2{,}106}{2{,}860}$	=			15·88 atm.
∴ Turgor pressure at saturation (= osmotic pressure at satn.)	=			15·88 atm.
∴ Original turgor pressure $= \frac{15{\cdot}88 \times (2{,}380 - 2{,}106)}{(2{,}860 - 2{,}106)}$	=		5·77 atm.	
Calculated original suction pressure = O.P. − T.P. = 19·05 − 5·77	=		13·28 atm.	
Observed value (above)	=		8·7 atm.	
Discrepancy = 13·28 − 8·7	=		4·58 atm.	

In making a first recalculation from Lyon's data it will be assumed that the aggregate vacuole volumes in Lyon's material amounted to half the total volume of tissue. This appears to be a fair estimate for a material like potato in which such a large proportion of the cell volume is occupied by starch: it may even be too great. It is evident that determinations would have to be made of the actual proportion of the block volume which took part in osmotic volume changes. Such determinations would probably be made, for the sake of a more stable basis of reference, not upon original material in an arbitrary condition but upon saturated material mounted in water. It will therefore be assumed for the purposes of calculation that the vacuoles in Lyon's material represented one-half of the total volumes of the blocks in the saturated condition. It will further be assumed that the principles proved above for spherical cells apply closely enough to polygonal cells and therefore that the symbols R and r for the flaccid radius and increment of radius on swelling of a spherical cell may be applied equally well to any linear dimension (e.g. length) of a cell and its corresponding increment on swelling, or therefore to the length and increment in length of a whole block. The blocks will be assumed to swell to the same relative extents as individual cells. Lyon's data are recalculated on these assumptions in tabular form in Table III, the format corresponding for ease of comparison with that of Table II.

TABLE III

Recalculation from Lyon's Data

		At incipient plasmolysis.	In original condition.	At saturation.
Measured suction pressures	=	21·5	8·7	0·0 atm.
Measured lengths of blocks	=	0·595	0·619	0·658 cm.
Calculated volumes of blocks	=	2,106	2,380	$2{,}860\times 10^{-4}$ cm.^{3}
If vacuole volumes = half total volumes at saturation, half the saturated volume (2,860/2) or 1,430 units remains constant.				
∴ Vacuole volumes (total volumes −1,430)	=	676	950	$1{,}430\times 10^{-4}$ cm.^{3}
∴ Ratios of osmotic pressures	=	1,430 :		676
	and	950 :	676	
Osmotic pressure at saturation				
$=\frac{676}{1{,}430}$ of O.P. at incipient plasmolysis				
$=\frac{676}{1{,}430}\times$ suction pressure at incipient plasmolysis				
$=\frac{676}{1{,}430}\times 21{\cdot}5$ atm.	=			10·12 atm.
∴ Turgor pressure at saturation (= O.P.)	=			10·12 atm.
Ratios of linear dimensions (from measured lengths)	=	100 :	104 :	110·5
Whence increments of linear dimensions, r	=	0	4	10·5
∴ Turgor pressures $=\frac{Kr}{R+3r}$	=	0	$\frac{4K}{112}$	$\frac{10{\cdot}5K}{131{\cdot}5}$
∴ $\frac{10{\cdot}5K}{131{\cdot}5}=10{\cdot}12$, whence $K=126{\cdot}8$				
∴ Original turgor pressure = $\frac{4\times 126{\cdot}8}{112}$	=		4·54 atm.	
The original osmotic pressure				
$=\frac{676}{950}$ of the osmotic pressure at incipient plasmolysis				
$=\frac{676}{950}\times 21{\cdot}5$	=		15·3 atm.	
Original suction pressure = O.P.−T.P. = 15·3−4·54	=		10·76 atm.	
Observed value (from above)	=		8·7 atm.	
∴ Discrepancy = 10·76−8·7	=		2·06 atm.	

The recalculation shows that when the true relation between turgor pressure and extension and an estimate of the relative vacuole volume are taken into account, the discrepancy of 4·58 atm. obtained by Lyon and taken as evidence for a non-osmotic force may fall to less than half its value. This is reproduced as the first value obtained by this method of recalculation founded on the first guess at the relative vacuole volume. The relative vacuole volume obviously needs estimating, but meanwhile it may be noted that further calculations show that if the relative vacuole volume be taken as 40 per cent. the discrepancy becomes only 0·66 atm., and if taken as about 38 per cent. there is no discrepancy at all, the calculated agreeing with the observed value. At a relative vacuole volume of 33 per cent. the discrepancy becomes 1·7 atm. the other way on, the observed value being the greater. Any of these relative vacuole volumes is equally possible. It may be noted that the elasticity modulus need not be known, but that unless the relative vacuole volume is known and taken into account the data mean nothing. Although the writer is in no way anxious to disprove the existence of non-osmotic forces in cell water relations, it must be agreed that these findings considerably alter the complexion of Lyon's work when presented as evidence for the action of non-osmotic forces in the water relations of potato tissue. It is hoped to repeat this type of experiment side by side with determinations of relative vacuole volumes.

I am very grateful to Professor W. H. Pearsall and Professor D. Thoday for helpful comments.

SUMMARY

It is shown that the relation between cell extension and turgor pressure for isotropic cells obeying Hooke's Law is not linear but hyperbolic. There can be no approximate linear relationship to any cell dimensions satisfactory for purposes of calculating turgor or osmotic pressures.

In calculating osmotic pressures of saps at different cell extensions it is important to use the ratios of volumes of the vacuoles only and not those of whole cells or blocks of tissue. This applies to all calculations of natural osmotic pressures from observed osmotic values.

Recalculations from existing data show the profound effect of taking these principles into account. In particular the data of Lyon are shown to provide no evidence for a non-osmotic force in potato tubers.

Determinations of the relative vacuole volumes should always be made and used when osmotic pressures are calculated from volume ratios. The hyperbolic relation of turgor pressure to the linear dimensions of the cells must also be taken into account.

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Studies in the Genus *Fucus*

II. The Anatomical Structure and Chemical Composition of Receptacles of *Fucus vesiculosus* from Three Contrasting Habitats[1]

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With four Figures in the Text

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INTRODUCTION

SEASONAL variations occur in the chemical composition of many brown seaweeds (Lapicque, 1919; Lunde, 1940; Dillon, 1943; Black, 1948), but no attempt has been made to correlate these variations with seasonal activities such as periods of active growth and reproduction. In *Fucus vesiculosus* the fertile regions of the thallus are sharply defined and form swollen receptacles at the apices of lateral branches. Their development proceeds over a period of several months, and collections of these fertile tips have been made at different developmental stages, to study the changes both in anatomical structure and in chemical composition which take place during receptacle development. Sterile tips were collected from the same plants at the same time as fertile tips,

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[1] This investigation formed part of a thesis accepted for the Degree of Doctor of Philosophy in the University of London, 1948.

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so that any seasonal variations in the chemical constituents distinct from those connected with fruiting would become apparent.

Vegetative plants of *Fucus vesiculosus* collected from contrasting habitats have been shown to exhibit differences in both anatomical development and chemical composition (Moss, 1948). The influence of environment is again considered in relation to anatomical development and chemical composition of receptacles from the same three habitats. These were selected on account of their varying degrees of exposure to wave action, which may result in further differences such as variations in mineral supply.

Although early stages in the development of the conceptacles of *Fucus vesiculosus* have been studied (Bower, 1880; Roe, 1916; Nienburg, 1913), the structure and development of the receptacle as a whole has received little attention.

Development and Anatomical Structure of a Receptacle

External morphology

The receptacles of *Fucus vesiculosus* are borne at the apices of lateral branches, which have dichotomized at frequent intervals compared with the longer internodes of the vegetative leaders (Knight, 1947). Externally the young receptacle is first apparent as a slight prolongation of the midrib from the thallus apex, with no development of the wings. Even in the mature receptacle the distinction between midrib and wings, which is so marked a feature of the vegetative thallus of *Fucus vesiculosus*, is not apparent.

Apical growth

During the very early stages of receptacle development a large apical cell lies at the base of the apical groove, similar to that first described by Woodworth (1888) at the vegetative apex. As the receptacle enlarges and becomes 3–4 mm. in length, the apical groove flattens out, and in the half-grown receptacle it is replaced by a convex tip.

During flattening out of the apical groove the identity of the single large apical cell becomes obscured. The convex tip of a mature receptacle is occupied by small compact cells surrounded by meristoderm. After the apical cell ceases to function, diffuse growth takes place throughout the receptacular tissues, accompanied by the deposition of mucilage. This diffuse growth in the fertile tips is maintained only during gamete maturation, for as soon as the gametes have been liberated, necrosis of the receptacular tissues sets in.

The meristoderm

The meristoderm is a tissue of maximum importance in receptacle development, for it is from this layer that ultimately the conceptacles are derived (Nienburg, 1913). In the young receptacle the cells of the meristoderm are brick-shaped with their longitudinal walls adjacent. Rapid surface growth accompanies enlargement of the receptacle, and the cells of the meristoderm undergo repeated anticlinal divisions. When seen in surface view they appear associated in groups of two to eight cells, representing products of recent segmentations. In the very young receptacle periclinal divisions also take place in the meristoderm and the segments cut off towards the interior develop into the cortex of the mature receptacle (Fig. 1). By the time of gamete extrusion the meristoderm has lost its meristematic activity and the cells are isodiametric. This loss of meristematic activity may be associated with the decay of receptacles after gamete extrusion.

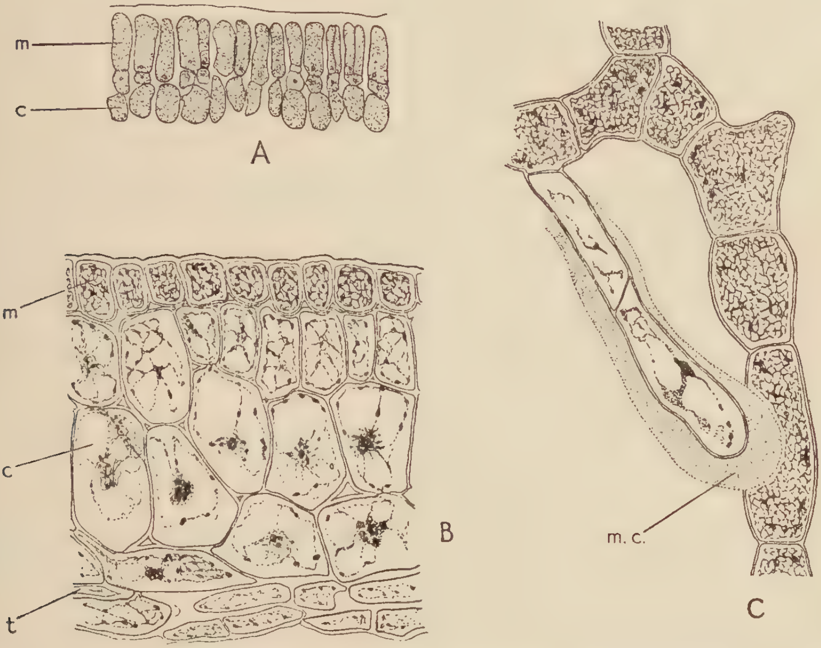

FIG. 1. A and B. Development of cortex in receptacles of *Fucus vesiculosus*. A in young receptacle, B in mature receptacle.

× approx. 500. *c* = cortex.
m = meristoderm *t* = layer of thick-walled cells.

C. Branching of medullary filament in receptacle of *Fucus vesiculosus*. × approx. 500.
m.c. = mucilage sheath.

The cortex

During secondary differentiation of the vegetative thallus of *Fucus vesiculosus*, secondary cortex originates as a result of both radial and tangential divisions in the cells of the primary cortex. But in the mature receptacle no secondary cortex is formed. As active surface growth of the receptacle is taking place, the original cortical initials laid down near the apex enlarge and become pigmented.

Behind the vegetative apex cortex grades into the central medulla, but in the receptacle there is a layer of thick-walled cells sharply separating these two tissues. These thick-walled cells are similar to those figured and described as 'mechanischen Systems' in *Himanthalia lorea* (Wille, 1910). Close to the apex cell-division in this layer is irregular, and a small-celled tissue with few intercellular spaces is laid down. The thickening of the cell-walls is uneven, and gives an immediate blue reaction with iodine and concentrated sulphuric acid, indicating the presence of cellulose. To keep pace with the rapid surface growth during receptacle enlargement, these thick-walled cells get stretched, especially in the longitudinal direction (Fig. 1, C). Although they are differentiated early in receptacle development, this layer of thick-walled cells is not continued into the thallus immediately beneath the receptacles.

The central medulla

In the very young receptacle, where the apical groove is still present, there is equal deposition of mucilage with separation of the filaments throughout the medulla. Progressive increase in the deposition of mucilage takes place from the outer medulla towards the centre, until the centre of a mature receptacle is occupied by mucilage traversed by a network of medullary cells. These remain in contact through simple pit connexions, similar to those developed in the vegetative part of the thallus.

Even in young receptacles the medullary cells branch freely in all directions, while elsewhere in the vegetative thallus branching of medullary cells is rare. The branches arise as lateral outgrowths from the original medullary cells, and are often protected in a thick envelope of mucilage (Fig. 1, C). Within the receptacle the medullary cell-walls remain thin, whereas in the older parts of the vegetative thallus they become extremely thick.

Secondary hyphae

In the vegetative apices hyphae have not been found closer to the apex than 4 mm., whereas in the mature receptacles they are present at the extreme tip. They originate even from the small cells at the extreme tip, and grow horizontally before they eventually turn down into the tissues of the receptacle. Elsewhere in the mature receptacle hyphae run in all directions through the intercellular mucilage, in contrast with the sterile tips where hyphae are directed toward the attaching disc. At the base of mature receptacles hyphae, originating from the sterile tissues below, grow up into the fertile region. In old receptacles, after gamete extrusion, hyphae have also been seen growing out from cells comprising the conceptacle wall. Secondary hyphae probably develop in the receptacles of many species of the Fucales, but their presence seems to have been noted hitherto only by Wille (1910) in *Himanthalia lorea* and Laing (1941) in *Bifurcaria laevigata*.

COMPARISON OF RECEPTACLES FROM THREE CONTRASTING HABITATS

Receptacle development proceeds over a period of several months. From each of the three localities, Cullipool (with strong exposure to wave action), Loch Melfort (sheltered), and Aird's Point (moderate exposure), receptacles were at similar developmental stages when collections were made during the same month.

In December the young receptacles which were collected for chemical analysis were up to 8 mm. in length and 3 mm. in breadth at the base, tapering to a convex tip. At this early stage they were surrounded by a very active meristoderm. As little intercellular mucilage had developed, a central strand of medullary cells was still present and few secondary hyphae had developed. In these young receptacles in December there was a gradation in conceptacle development from the base to the apex, the oldest conceptacles being differentiated at the base. These already contained numerous antheridia or oogonia. In the latter two to eight nuclei were visible, although none showed cleavage into distinct ova. At the same time, the conceptacles at the tips of these receptacles were very small and neither oogonial nor antheridial initials were visible.

Mature receptacles were collected from each locality in June, those from Cullipool (exposed habitat) being much larger than receptacles from either Loch Melfort or Aird's Point. In spite of differences in size both the anatomical structure and developmental stage of antheridia and oogonia were similar from the contrasting habitats. In these mature receptacles the conceptacles were full of ripe gametes from the base to the extreme tip. Firm mucilage traversed by a network of medullary cells filled the centres of these receptacles, and secondary hyphae meandered in all directions.

Two months later, in August, receptacles from all localities had shed most of their gametes. The conceptacles contained numerous exo-chitons, and sometimes a few young oogonia or antheridia along the base of the conceptacle wall. At this late stage most of the intercellular mucilage had been lost with gamete extrusion, and many of the receptacles had been damaged, probably by waves or browsing animals.

In vegetative parts of well-grown thalli it has been observed that cortical and hyphal development is most marked in plants from the open sea (Moss, 1948). The mature receptacles, however, of plants from the open sea were about twice the size of those from the other two habitats but were remarkedly similar as to development of cortex and hyphae. But the receptacles are only temporary structures, disintegrating as soon as the gametes have been discharged, and this may account for the apparent lack of formative response to contrasting environmental factors.

CHEMICAL COMPOSITION OF RECEPTACLES FROM THE SAME THREE HABITATS

Collection of material

The first collections were made between June 3 and June 13, 1947, when receptacles were mature. A second series of collections was made between July 30 and August 3, 1947, i.e. approximately 7–8 weeks later, when most of the gametes had been shed and the receptacles were beginning to disintegrate.

Between December 5 and 12, 1947, a third series of collections was made, when new receptacles were at a very early stage of development. This latter series will be referred to as stage I in relation to receptacle development, while June samples represent stage II and August samples stage III in this developmental series. These numbers are used in the tables of analytical results.

At each collection approximately 100 lb. of fruiting plants were gathered from each of the three localities. *Fucus vesiculosus* is dioecious and after the random collection, male and female plants were separated. Samples were then cut from each thallus as shown in Fig. 2; samples from each sex being kept separate. Samples C were taken only in June when the receptacles were mature. The small weight of December samples necessitated the mixing of both male and female receptacles from Aird's Point, and of sterile tips of male and female thalli from both Aird's Point and Cullipool. As the samples were cut off individually by hand, any tips with epithytic plant growths or attached Polyzoans were discarded. Also any receptacles on a thallus which were obviously much younger or older than the average selected for that particular sample were not included.

The samples were dried on racks above a coke furnace at the Oban outstation of the Scottish Seaweed Research Association, and afterwards ground to a fine powder in a Christy and Norris No. 8 laboratory mill fitted with a $\frac{1}{64}$-inch perforated plate screen. It was calculated that in June approximately 7,650 receptacles or 2,500 sterile tips were included in each sample, while in December some 11,000 receptacles were collected from each locality. The analytical methods used throughout are those developed by the Scottish Seaweed Research Association (Black, 1948; Cameron, Ross, and Percival, 1948).

Data obtained from fertile plants collected at Aird's Point on June 3, 1947, showed that there was an average number of 47 sterile tips to 125 fertile tips per thallus. The average fresh weight of a thallus was 87·5 g., of which the receptacles alone accounted for 49 g., i.e. more than half the total fresh weight of a fertile plant. This is of technical interest, for if harvesting of the weed for industrial purposes occurs during the reproductive period, then a greater weight of unwanted water will be included in the freshly gathered plants.

Percentage dry weight

It is difficult to obtain an accurate estimation of the percentage dry weight of parts of a thallus of *Fucus vesiculosus*. Being an intertidal species, the plants are naturally collected at low tide. At times of bright sun or drying wind their tips are often dry and crisp, and the older parts will also have lost a considerable amount of water through exposure to the atmosphere (cf. Pringsheim, 1923; Isaac, 1933; Zaneveld, 1937). In order to compensate for this loss, the material to be dried was previously soaked for 30 minutes in sea-water from the same locality as the plants.

In Table I the results obtained from samples collected at Aird's Point (moderate exposure) are summarized. Material from both Loch Melfort (sheltered shore) and Cullipool (open sea) gave similar values.[1]

TABLE I

Dry Weight (expressed as Percentage Fresh Weight)

		Stage I, Dec.	Stage II, June.	Stage III, Aug.
Male	Receptacles	16·9	11·2	9·9
	Below receptacles	—	—	—
	Sterile tips	20·5	24·9	22·0
Female	Receptacles	16·9	11·8	8·8
	Below receptacles	—	27·3	—
	Sterile tips	20·5	24·6	26·9

From plants collected at the same time, the percentage dry weights of sterile tips from both male and female plants are similar. There is also a seasonal variation in the dry weights of sterile tips. In the observations recorded higher values were obtained in August than in June, and December, or September samples (Moss, 1948). Walker (1948) has shown that whole thalli of *Fucus vesiculosus* give quite different results, the percentage dry weights being low during the summer months. A probable explanation of this is put forward later.

Receptacles of both sexes at similar developmental stages have similar dry weights. Even at a very early developmental stage the water content of receptacles is higher than in sterile tips of the same thalli graph I, Fig. 3. As the intercellular mucilage increases with maturation of the gametes, so does the water content increase. After the gametes are shed, most of the intercellular mucilage has been lost too, but the interior of the receptacles becomes filled with water so that low percentage dry weights were still obtained for receptacles collected in August.

The increase in the uptake of water accompanying maturation of the reproductive organs is localized in the receptacle itself, for when a low value of 12·3 per cent. is given for Loch Melfort male receptacles in June, the part of the thallus immediately beneath the receptacles gives a value of 26·3 per cent.; some 3 per cent. higher than the value for sterile tips from the same plants.

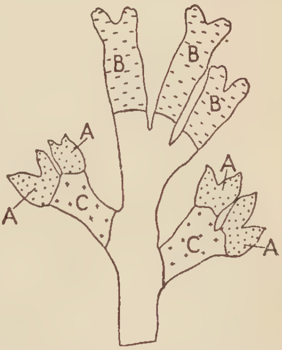

FIG. 2. Diagram showing samples of *F. vesiculosus* collected for chemical analysis.

A = Receptacles.

B = Sterile tips (approximately 1–1½ inches long).

C = Thallus below receptacles (approximately 1–1½ inches long).

The results summarized in Table I are interesting to compare with the seasonal variation in the percentage dry weights of *whole thalli* of *Fucus*

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[1] Complete tables of results of chemical analyses of material from both Loch Melfort and Cullipool are tabulated in thesis for Ph.D. degree (London) 1948.

vesiculosus, recently published by Walker (1947). His plants were collected each month from Aird's Point near Port Appin, i.e. from the same locality as some gathered for the present investigation. He states that the percentage dry weight of 'Each species (i.e. *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosus*, and *Fucus spiralis*) rises and falls twice each year. It will be recalled that the diatom cycle rises and falls twice each year'. The rise and fall twice each year is not so marked in *Fucus vesiculosus* as in *Fucus spiralis*.

It was in June and August 1947 that fertile thalli of *Fucus vesiculosus* were collected from Aird's Point for the present investigation, and the dry weights of the receptacles were very low, approximately 10 per cent. As previously stated, the receptacles at this season constitute over half the total fresh weight of a thallus. Hence their effect would be to lower the percentage dry weight of a complete thallus, and it is the presence of mature receptacles on the thalli which accounts for the low values reported by Walker for July 1945 and 1946. For a previous investigation (Moss, 1948) *Fucus vesiculosus* had also been collected from Aird's Point in September 1946. At this season no fertile plants were found. Hence if thalli are devoid of fertile tips in autumn, it follows that percentage dry weights of complete thalli would be higher than when they are bearing swollen receptacles with high water content. It is at the non-fruiting period that Walker reports the highest percentage dry weights for 1946. Thus the seasonal variation in dry weights of complete thalli of *Fucus vesiculosus* from Aird's Point described by Walker can be related to the reproductive cycle of that species. The relationship to the diatom cycle is obscure.

Walker's figures for September and October are similar to those recorded for the same months for material collected at Cove and Stonehaven by Hendrick (1898), 32·09 per cent. and 35·05 per cent. respectively. Butler (1931) gives the dry weight of *Fucus vesiculosus* collected from St. Andrews, New Brunswick, in June and July as 23·6 per cent. Unfortunately the period of reproduction in this locality is not given, but this value is much higher than that obtained by Walker for plants collected off the west coast of Argyllshire during the same months.

It has been shown that a decrease in the water content of the tissues occurs from the apex to the base of a vegetative thallus of *Fucus vesiculosus* (Moss, 1948). But nowhere in the sterile thallus was there such a great difference in the water content as has now been shown to exist between the tissues of mature receptacles and sterile tips from the same plants. Moreover, at the same time water content is decreasing in certain tissues, as in the sterile tips during summer, in other parts of the thallus it may simultaneously be increasing, as during the maturation of receptacles.

Percentage total ash

The results recorded in Table II are for samples collected from Aird's Point, and very similar values were obtained for material from both Loch Melfort and Cullipool. They show extremely high values for total ash, ranging from 15·6 per cent. for Loch Melfort sterile tips in August to 40·0 per cent. for receptacles from the same plants. A survey of the literature shows that high values for the ash content of *Fucus vesiculosus* are reported elsewhere (presumably for complete thalli): 20·78 per cent. (Hendrick, 1898), 20·07 per cent. (Butler, 1931), 22·00 per cent. (Lunde, 1941).

TABLE II

Total Ash (expressed as Percentage Dry Weight)

		Stage I, Dec.	Stage II, June.	Stage III, Aug.
Male	Receptacles	25·5	35·5	37·3
	Below receptacles	—	19·5	—
	Sterile tips	23·5	18·6	17·8
Female	Receptacles	25·5	35·5	37·3
	Below receptacles	—	20·7	—
	Sterile tips	23·5	18·9	18·0

Sosa-Bourdouil (1940) found low percentages of ash in the gametes, 8·5 per cent. in the antherozoids, and 5·4 per cent. in the ova on an anhydrous basis. Thus the high ash content of mature receptacles is not associated with the gametes but with the receptacular tissues. It is interesting to note that Dawson Turner (1809) says: 'In the islands of Jura the inhabitants dry their cheeses without salt, by covering them with the ashes of this plant which abounds with such quantities of salts, that from 5 ounces of the ashes may be procured 2½ ounces of fixed alkaline salts.' The results here recorded in Table II on a dry weight basis show enormous differences between sterile and fertile tips in June and August, the fertile tips containing approximately twice the percentage of total ash as do the sterile tips from the same plants.

Lapicque (1919) found that the percentage ash of *Laminaria flexicaulis* was high in winter and low in summer. Soluble carbohydrates were low in winter when ash was high, and high in summer when ash was low. Hence he said, 'il est plausible qu'il s'agisse simplement d'une substitution isotonique'. Lapicque's calculations were on a dry-weight basis, and on a similar basis these results for *Fucus vesiculosus* show great variations during receptacle development, ash being low in December samples and high in June and August. But if calculations of the ash content are on a fresh-weight basis, then comparatively little variation takes place during receptacle development, graph II, Fig. 3. It is these calculations on a basis of fresh weight which are essential for a comparison of the chemical composition of the actual plants which are analysed.

The percentage ash on an anhydrous basis shows as marked a variation in relation to receptacle development as do any of the organic constituents. As the water content increases with maturation of the receptacles, so does the ash content in relation to the increase of other constituents.[1] At the same time the part of the thallus immediately below the mature receptacles has an ash content similar to that of the sterile tips. So that the high percentage ash on an anhydrous basis correlated with a high water content of the mature receptacles is confined to the fruiting body and does not affect the thallus beneath it, from which it differs so much in anatomical organization.

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[1] Since sending to press, it has been ascertained that this increase is mainly in water soluble constituents, especially soluble chlorides.

Percentage organic nitrogen

The total organic nitrogen was determined by the standard Kjeldahl method, and the result multiplied by 6·25 gives the percentage crude protein. In Table III are the data obtained for samples from Aird's Point. Those from Loch Melfort and Cullipool show similar variations, although the values given for Loch Melfort (sheltered habitat) are always lower than those for corresponding samples from either of the other two localities.

TABLE III

Organic Nitrogen (expressed as Percentage Dry Weight)

		Stage I, Dec.	Stage II, June.	Stage III, Aug.
Male	Receptacles	2·08	1·39	0·88
	Below receptacles	—	1·47	—
	Sterile tips	1·58	1·72	0·85
Female	Receptacles	2·08	1·05	0·63
	Below receptacles	—	1·04	—
	Sterile tips	1·58	1·70	0·93

At each developmental stage from either of the three habitats a higher percentage of organic nitrogen is present in the male than in the female receptacles. Sosa-Bourdouil (1940) found very high nitrogen content in the gametes of *Fucus vesiculosus*: 8·5 per cent. and 4·7 per cent. for antherozoids and ova respectively. The higher percentage of nitrogen in the male gametes may account for the slightly higher values always obtained for male receptacles.

Between June and August the organic nitrogen content of all samples is halved. The value of taking both sterile and fertile tips from the same plants is here very apparent, for if only fertile tips had been collected, then one might conclude that the decrease from June to August accompanied liberation of the gametes, especially as these have very high nitrogen content. But the parallel decrease in the organic nitrogen content of sterile tips shows that in these parts of the thallus there is a seasonal variation in organic nitrogen content. It is high in winter and early summer with a rapid decline in late summer, a variation distinct from and not related to fruiting. It is probably related to the supply of available nitrogen.

The great variation in organic nitrogen found between similar samples of thalli collected in June and August emphasizes the necessity of stating the time of collection of plants if analytical results are to have any scientific value. Different seasons of collecting probably account for the great differences for the organic nitrogen content of *Fucus vesiculosus* reported: 2·47 per cent. (Hendrick, 1898), 2·43 per cent. (Vincent, 1924), 2·29 per cent. (Barlow, 1911), 1·61 per cent. (Wheeler and Hartwell, 1893), 1·37 per cent. (Butler, 1931). Butler's figures are for material collected from New Brunswick in July, and as they are presumably for complete plants, they cannot be directly compared with the present results. These show that organic nitrogen of mature receptacles is low, so that during the period of fruiting the receptacles would lower the value obtained for a complete plant.

Calculations on a fresh-weight basis show that when *Fucus vesiculosus* is fruiting there is always a higher percentage of protein in the vegetative tips of thalli than in the fertile tips of the same plants, graph III, Fig. 3. The greatest difference occurs at maturation of the gametes, when there is three to four times as much protein in the sterile tips as in the receptacles. At the same season the region of thallus immediately beneath the receptacles gives a value intermediate between that of the receptacles and the sterile tips.

Percentage mannitol

The data obtained for samples collected from Aird's Point are set out in Table IV. Material from Loch Melfort and Cullipool showed similar variations in mannitol content, although the values from both the sheltered and exposed habitat were slightly higher than those from Aird's Point.

TABLE IV

Mannitol (expressed as Percentage Dry Weight)

		Stage I, Dec.	Stage II, June.	Stage III, Aug.
Male	Receptacles	8·65	5·55	5·46
	Below receptacles	—	11·93	—
	Sterile tips	10·02	12·59	15·0
Female	Receptacles	8·65	5·88	5·73
	Below receptacles	—	11·43	—
	Sterile tips	10·02	12·77	16·34

These results, whether calculations are on dry or fresh weight basis, show that there is at all stages of receptacle development a lower percentage of mannitol in the receptacles than in the vegetative tips from the same thalli, the greatest difference occurring in August when most of the gametes have been shed. While calculations on an anhydrous basis show for August samples approximately a 1:3 ratio of percentage mannitol in receptacles to percentage mannitol in vegetative tips, calculations on a fresh weight basis give a ratio of 1:4 for the same samples.

The percentage mannitol in the sterile tips increases from December to August, at the same time as it is decreasing in the receptacles (Fig. 4). This indicates that mannitol is not a storage material of either the ova or antherozoids of *Fucus vesiculosus*. Mannitol is at its maximum when organic nitrogen is at its minimum. The region of thallus immediately below the mature receptacles in June has a lower percentage mannitol than the vegetative tips, but a much higher value than receptacles from the same thalli. Thus the very low percentage of mannitol is located within the receptacle itself. The seasonal variation in the mannitol content of the vegetative apices of thalli of

Fucus vesiculosus is similar to the seasonal variations hitherto recorded for species of *Laminaria* (Lunde, 1937; Dillon, 1940; Black, 1948).

Percentage alginic acid

Table V records the alginic acid content of samples collected from Aird's Point. Similar variations were shown in material from both Loch Melfort and Cullipool. although sterile tips of plants from the sheltered locality (Loch Melfort) always gave lower results than those from habitats with moderate or strong exposure to wave action.

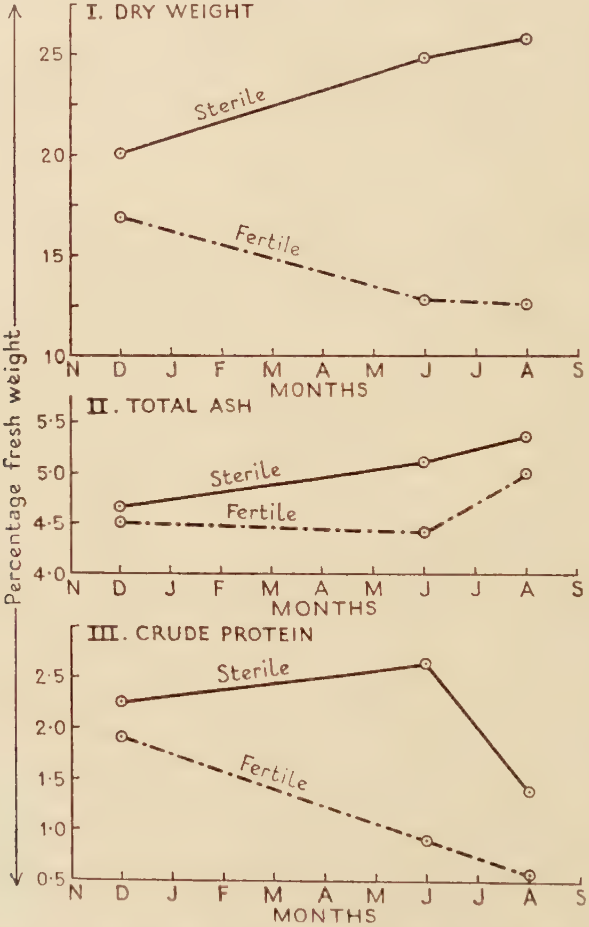

FIG. 3. Variations in dry weight, total ash and protein content (expressed as percentage *fresh weight*), of sterile and fertile tips of female thalli of *Fucus vesiculosus* from Cullipool.

Estimations of the alginic acid content of various species have been determined, and in species of *Laminaria* it has been demonstrated that a seasonal variation in alginic acid occurs (Lunde, 1937; Dillon, 1943; Black, 1948). These investigators report that the maximum percentage of alginic

TABLE V

Alginic Acid (expressed as Percentage Dry Weight)

		Stage I, Dec.	Stage II, June.	Stage III, Aug.
Male	Receptacles	26·99	18·53	19·32
	Below receptacles	—	17·12	—
	Sterile tips	29·38	18·39	17·53
Female	Receptacles	26·99	18·01	18·22
	Below receptacles	—	16·46	—
	Sterile tips	29·38	18·28	17·42

acid in Laminaria is reached in spring. In samples of *Fucus vesiculosus* analysed a much higher percentage of alginic acid has been found in the vegetative tips of thalli in December than in either June or August. These December figures, 27·2 and 29·8 per cent. for Cullipool and Aird's Point respectively, are much higher than Lunde's figure previously given for *Fucus vesiculosus* (19 per cent.). This is presumably for complete plants, but previous results (Moss, 1948) have shown that there is a much higher percentage of alginic acid in the young vegetative tips than in other regions of the thallus of *Fucus vesiculosus*.

The greatest proportion of alginic acid is found in the sterile tips when ash and mannitol are lowest. The samples of thallus taken below the mature receptacles have a similar alginic acid content to sterile tips from the same thalli. Hence as noted previously for mannitol, the lower percentage alginic acid on fresh weight basis is confined to the fruiting body and does not affect the thallus immediately beneath it.

Fig. 4 shows the variation in alginic acid content of tips of thalli from Cullipool, calculated on fresh weight basis. In December the young receptacles have a lower percentage alginic acid than have the vegetative tips at the same season, and this decreases rapidly as the receptacles mature, and falls still farther when most of the gametes have been shed.

Unestimated residue

Although there are great variations in the chemical composition of sterile and fertile tips of the thalli collected at the same season, yet the total estimated substances in the present investigation account for similar percentages of the dried samples of both. In June and August samples 55–60 per cent. of the dried weed has been estimated, whereas the same methods account for 65 per cent. of the dried weed collected in December. This shows that in all samples there is a residue of 30–40 per cent. of the dried weed to be estimated. Preliminary analyses show that laminarin and fats constitute some of this residue.[1]

The doubling of the ash content during development of receptacles (on dry weight basis) is an interesting phenomenon, and an analysis of this ash would yield information as to the nature of this mineral accumulation associated with reproduction in *Fucus vesiculosus*.

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[1] It has since been found that both laminarin and ether-soluble materials decrease during development of receptacles, so that less than 2 per cent. of each were found in mature receptacles from all habitats, on dry weight basis.

GENERAL CONSIDERATIONS

The anatomical structure and chemical composition of the receptacles of *Fucus vesiculosus* have been found to be very similar in the three contrasting habitats selected, whereas the vegetative thalli exhibited marked differences (Moss, 1948). The sterile tips which were collected for this investigation show a much higher percentage of alginic acid in plants from the open sea than from the sheltered loch.

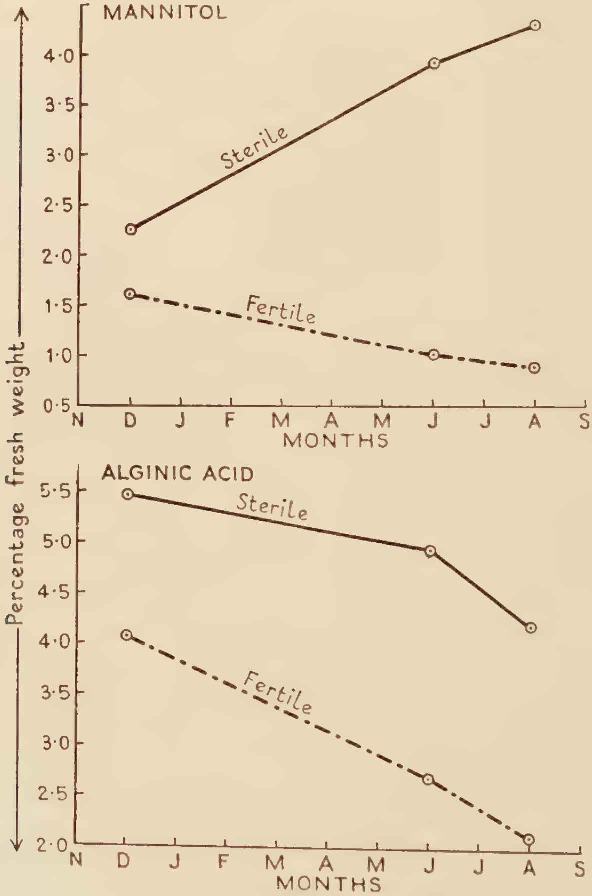

FIG. 4. Variations in mannitol and alginic acid content (expressed as percentage *fresh weight*) of sterile and fertile tips of female thalli of *Fucus vesiculosus* from Cullipool.

At the initiation of a receptacle there is a change in the metabolism of the sterile tip correlated with the anatomical changes which are also taking place.

Fucus vesiculosus is dioecious; water, ash, mannitol, and alginic acid content of plants of both sexes are similar, but organic nitrogen is slightly but consistently higher in male than in female receptacles.

Analyses of sterile tips taken from the same thalli as receptacles show that seasonal variations in chemical composition occur, differing from and even varying in the opposite direction from those connected with reproduction. Mannitol, for example, has a low value in December, reaching a maximum during late summer. In contrast, in the receptacles, mannitol is lowest in late summer. Since the receptacles may form more than half the fresh weight of a plant, this emphasizes the importance of considering the growth and reproductive cycle in connexion with variations in chemical composition of whole thalli of seaweeds throughout the year; an aspect hitherto ignored amongst the vast literature on the chemical composition of seaweeds.

ACKNOWLEDGEMENTS

This investigation was carried out in the Department of Botany, Westfield College, during the tenure of a Postgraduate Research Studentship of the University of London. I wish to express my sincere thanks to Dr. E. M. Delf for assistance with the preparation of this paper for publication.

I wish to thank the Director and Staff of the Scottish Seaweed Research Association for their help and generosity in providing facilities for collecting the material used in this investigation.

SUMMARY

Stages in the development of receptacles of *Fucus vesiculosus* have been collected from three contrasting habitats off the west coast of Argyllshire, for a study of their anatomical structure and chemical composition.

No marked differences in development, anatomical structure, and chemical composition have been found in receptacles from these three contrasting habitats. They are temporary structures concerned with the period of reproduction. It is in the perennial vegetative parts of the plant that the contrasting environmental conditions of sheltered loch and open sea had more pronounced effects, both upon anatomical structure and chemical composition.

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Studies in the Genus *Fucus*

III. Structure and Development of the Attaching Discs of *Fucus vesiculosus*[1]

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With Plate XIV and three Figures in the Text

INTRODUCTION

THE basal organ in the Fucaceae consists generally of a small discoid structure adhering to the substrate, although in *Phyllospora* numerous branches like laminarian haptera arise from a central disc. *Fucus vesiculosus* occurs on a variety of substrates, and externally the attaching disc seems equally well developed whether it is on solid rock, iron girders, or wooden breakwaters. On any of these substrates it is secure enough to resist the constant strain to which the thallus is subjected by the movement of waves. Where thalli are not subjected to this constant strain, as on salt marshes, the basal organ of *Fucus vesiculosus* is not developed (Baker, 1912, 1916) and the plants are unattached. In a similar manner, the attaching discs of *Pelvetia canaliculata* (Baker, 1912) and *Ascophyllum nodosum* (Cotton, 1912) are absent from salt-marsh forms.

The combined features of dioecism and external fertilization have made *Fucus vesiculosus* a favourite organism for the study of polarity in relation to cleavage of the zygote (Nienburg, 1923; Whitaker, 1931, 1937, 1938). Thuret and Bornet (1878) give admirable figures of the early developmental stages of sporelings of *Fucus serratus*, *Pelvetia canaliculata*, and *Ascophyllum nodosum*. They say that *Fucus vesiculosus* resembles *Fucus serratus* in its development, but they did not trace the development of the basal organs beyond the rhizoids of the sporelings. Oltmanns' studies on fucoid sporelings (1889) and later those of Nienburg (1923) are in relation to thallus differentiation, and the classical accounts on the anatomical structure of the fucoids by Reinke (1876) and Hansteen (1892) do not trace out the developmental sequence of the attaching organs. The development of adventitious branches from the upper surface of the attaching disc is a common feature of *Fucus vesiculosus*, and their origin has been described by Oltmanns (1889).

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[1] This investigation formed part of a thesis accepted for the Degree of Doctor of Philosophy in the University of London, 1948.

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MATERIAL AND METHODS

For the early stages in the ontogeny of the attaching organs of *Fucus vesiculosus* sporelings were raised in culture, using natural sea-water, after boiling and filtering, as the culture medium. Packets of ripe oospheres and antherozoids were removed from the ostioles of discharging conceptacles with a fine brush and mixed together in a glass dish containing sea-water. Fertilized eggs sank to the bottom of the dish, and the following day early stages in sporeling development could be found. From sporelings cultured in this way development of the attaching organs could be studied for several weeks until the young sporelings became overgrown with diatoms and other micro-organisms.

Sporelings were also cultured on a substrate of pith. Discs of pith, about 3 mm. thick, were floated on a thin layer of melted paraffin wax in the bottom of a glass dish, so that on cooling the discs were firmly fixed to the dish. Care was taken to ensure that the top of the discs did not get covered with a layer of wax. Sea-water was then placed in the dish, and oospheres and antherozoids introduced and well mixed. The fertilized eggs settled on the discs of pith and there germinated.

Sporelings raised on pith remained relatively free from micro-organisms for a year, although they showed no further signs of development after 5 months when they were approximately 1·5 mm. in length. The sea-water in the culture dishes was changed about every 3 weeks, and the cessation of development might be due in part to starvation from certain nutrients. Moreover, the sporelings were maintained in culture under continuous immersion, and this may hinder the normal development of an intertidal organism. Also the culture dishes were kept near a laboratory window facing south-east, and as the culture was started in November 1946, after about 5 months' growth the young sporelings were exposed to very bright sunlight for part of each day. But these sporelings cultured on a substrate of pith remained relatively free from diatoms for a longer period than did those raised on a glass substrate. The rhizoids penetrated the pith cells and remained free of the diatoms which so often attacked the rhizoids of sporelings cultured on glass.

After 4 to 6 weeks' growth sporelings cultured on pith started to develop apical hairs, and by about 3 months each possessed an apical tuft of hairs, almost equal in length to the entire length of the rest of the sporeling. Small adventitious branches arose from the lower part of some of the sporelings after about 4 months' growth, and these also developed a tuft of apical hairs. After 6 to 8 months the apical hairs gradually fell off.

Some of these sporelings raised in culture were studied while still living, while others were fixed in formalin-acetic alcohol, sectioned and stained in gentian violet. The attachment of the sporelings to discs of pith made it easy to determine their orientation for embedding and sectioning.

For subsequent stages in the development of the attaching discs, both sporelings from the *Fucus vesiculosus* zone and adult plants were collected from a wooden breakwater at Cromer (Norfolk). Attaching discs of mature plants were also collected from rocks in a sheltered bay in Loch Melfort (Argyllshire). From south of Port Appin (Argyllshire) *Fucus vesiculosus*, growing upon *Ascophyllum nodosum* and *Fucus vesiculosus*, was obtained. These discs were fixed in formalin-acetic alcohol, and preparations were stained with gentian violet.

DEVELOPMENT OF THE ATTACHING DISC

Attachment of the sporeling

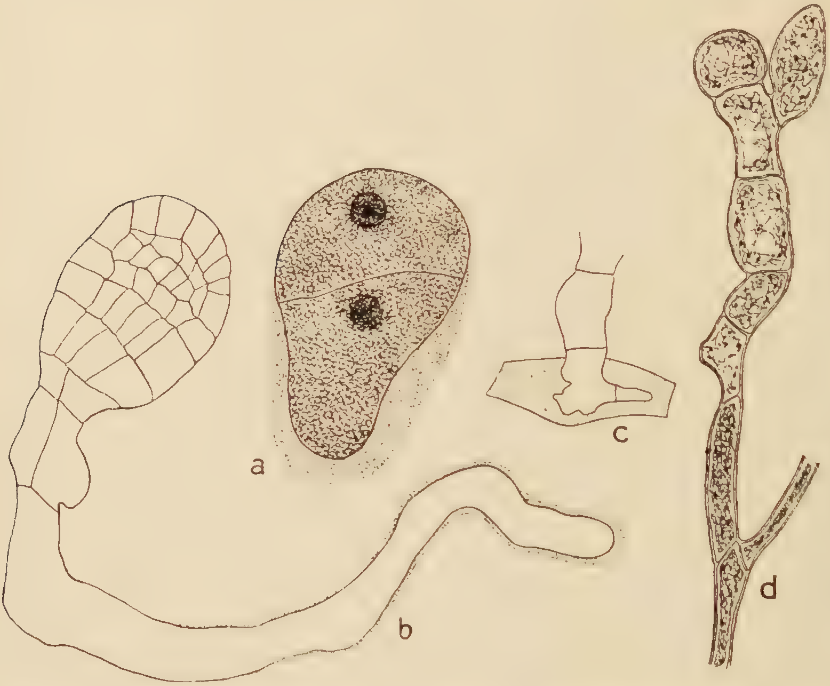
Germination of the zygote of *Fucus vesiculosus* follows soon after fertilization. Before there is any cleavage of the cytoplasm a small protuberance grows out from one side and forms the primary rhizoid. From its earliest appearance the primary rhizoid is surrounded by a thick mucilaginous envelope (Text-fig. 1 *a*). This mucilage secures the young sporeling to the substrate, for sporelings with only a slight rhizoidal protuberance surrounded by this mucilage strongly adhere to the bottom of a culture dish. This envelope of mucilage surrounding the primary rhizoid is clearly figured by Thuret and Bornet (1878) in sporelings of *Fucus serratus* and *Ascophyllum nodosum*. It is ignored in the later figures of Oltmanns (1889) and Nienburg (1923), although Oltmanns says (1922) 'Dieses besorgt alsbald die Festheftung und wird dabei unterstützt von den äussersten Membranschichten, welche stark verschleimen. Das ist auch später noch erkennbar'. The mucilage is transparent and easily overlooked under examination with bright light. However, in the early stages of germination the envelope of mucilage is very thick and is rendered more conspicuous with very dilute gentian violet. The mucilage readily takes up the stain, and if care is not exercised it becomes overstained, and the delicate membrane of the rhizoidal cell inside the mucilage layer gets obscured.

During the first 2 days of growth the primary rhizoid increases in length and a transverse wall cuts off the rhizoid from the upper cell (Text-fig. 1 *a*), which eventually gives rise to the thallus. Oltmanns (1922) figures a sporeling of this species with a much branched unicellular rhizoid, but I have never observed such branching in material cultured on a substrate of either glass or pith. The primary rhizoid increases in length until it is several times that of the rest of the sporeling. Thin, usually oblique transverse walls develop in the rhizoid so that a long filament of cells is formed, still surrounded by a thin layer of mucilage.

In the cell above the primary rhizoid, longitudinal divisions take place and protuberances from the cells thus cut off may develop into secondary rhizoids (Text-fig. 1 *b*). The tips of these rhizoids are also surrounded by a mucilaginous envelope. They undergo successive branching and many eventually become as long as the primary rhizoid, so that, in sporelings cultured on a substrate of glass, the identity of the primary rhizoid becomes obcured. When germinated on glass, the rhizoids of the sporelings are richly pigmented. After

3 to 4 weeks the mucilage envelopes of the rhizoids become heavily infested with micro-organisms.

When sporelings were germinated on discs of pith, the primary rhizoid grew down into the pith. It penetrated not only between the pith cells but also broke through the cell-walls and traversed the cell cavities. Subsequent rhizoids penetrated in a similar manner. Compared with the rhizoids growing on a glass substrate, those penetrating into pith were more richly branched. Contact with an obstacle appeared to stimulate branching, for as shown in Text-fig. 1 *c* when the apex of a rhizoid meets an obstacle (here a pith cell-wall) it swells up, becomes irregular in shape, and sends off branches in other directions where less resistance is encountered.



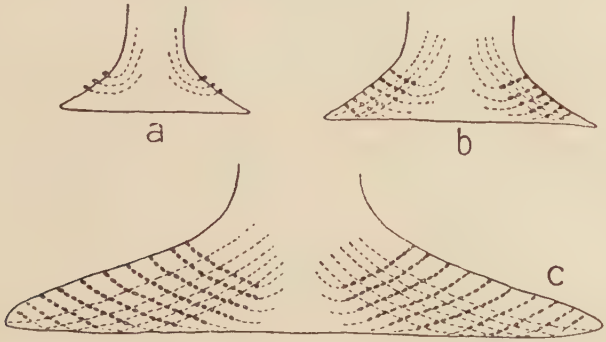
TEXT-FIG. 1. *a*. Early stage in development of sporeling of *Fucus vesiculosus*, showing mucilage sheath around the rhizoid. *b*. Development of secondary rhizoids from lower cells of sporeling. *c*. Branching at the tip of a rhizoid of a young sporeling. *d*. Upgrowing hyphal filament from the pseudo-cortex of a young disc.

With further development of the sporeling, hyphae arise from the lowermost cells of the young thallus and later from the internal tissues. Those hyphae arising from the innermost cells of the sporeling form very long narrow filaments which advance by apical growth through the tissues and eventually push their way down amongst the rhizoids. While some penetrate down into the substrate, others push their way to the surface, and spreading out over the rhizoids, begin to form a small attaching disc on the surface of the substrate.

This process is continued throughout the life of the plant. Those hyphae which penetrate to the outer limits of the disc continue to advance over the substrate and thus increase the area of the disc.

Attachment of the mature plant

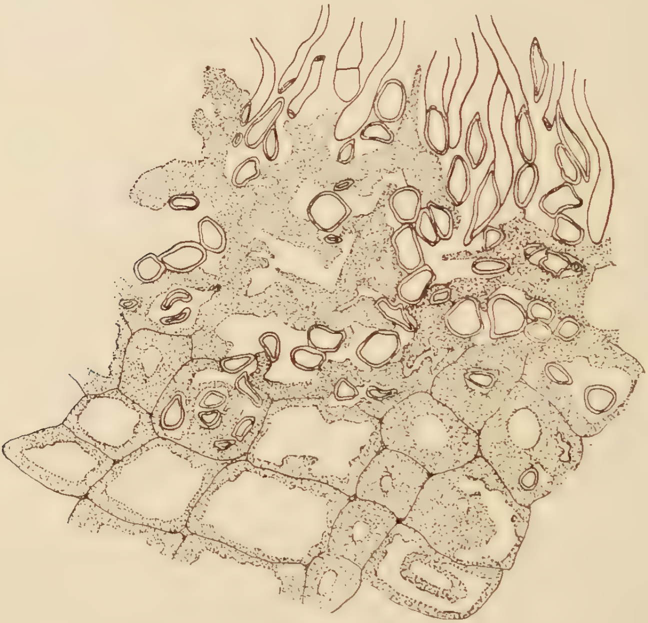
In the case of attaching organs of sporelings a few millimetres in length collected from a wooden breakwater at Cromer (Norfolk) hyphae had filled every available space in the eroded cells on the surface of the breakwater. In the disc of a sporeling about 5 mm. in length some of the hyphae coming in from the thallus interweave amongst the rhizoids and other descending hyphae, and then turn out till they reach the upper surface of the young disc. The interweaving of the hyphae in the disc resembles the warp and weft threads of a fabric, whereas in the stipe the hyphae intertwine like rope fibres.



TEXT-FIG. 2. Diagrams *a*, *b*, and *c* demonstrating development of the pseudo-cortex and parallel hyphal filaments in the disc of *Fucus vesiculosus*.

When the hyphae reach the upper surface of the disc they start to segment. Text-fig. 1 *d* shows such a hyphal filament teased from a young disc, which has produced a row of five cells at its tip. The rectangular form of these cells is in contrast with the very narrow elongated cells of the hyphae below. These cells cut off towards the exterior, having advanced beyond the mass of interweaving hyphae, enlarge, and become deeply pigmented. They are uninucleate and the outermost round off and get worn away so that the upper surface of the disc often has a layer of dead cells and debris over it. As can be seen in Text-fig. 1 *d*, the subterminal cell of an upgrowing hyphal filament frequently gives rise to a lateral branch. The close approximation of numerous of these upgrowing hyphal filaments eventually results in the formation of a 'pseudo-cortex' over the upper surface of the disc.

As soon as these upgrowing hyphal filaments have started to segment, further hyphae coming in from the thallus penetrate between them to the outer limits of the disc. The upgrowing filaments undergo further segmentation, and so continual hyphal penetration and further segmentation of the upgrowing hyphal filaments lead to the development of parallel filaments in the disc of a mature thallus (Text-fig. 2). The regularity of these filaments in the disc of a mature thallus is striking, for the cells comprising the parallel filaments are regular and rectangular in shape, and stand out in sharp contrast against the narrower and longer cells of the interweaving hyphae. Plate XIV, A, is a photomicrograph of part of the disc of a mature thallus showing the pseudo-cortex from which parallel filaments can be traced back into the disc. In a well-developed disc the hyphal filaments have cut off about a dozen cells beyond the zone of hyphal penetration and these form a pseudo-cortex over the upper surface of the mature disc. The filaments composing the pseudo-cortex may branch. Their cells are pigmented and the cell-walls remain thin, even in the parallel filaments amongst which hyphae have interwoven. In a well-developed disc these parallel hyphal filaments are absent from the immediate centre (Text-fig. 2) and this represents the position of the original attaching organ of the sporeling, before there had been any development of a pseudo-cortex. Advancement of the attaching disc over the substrate is the result of penetration of hyphae from the interior of the disc to its outer limits, so that all stages in development of the pseudo-cortex can be followed from the outer margin towards the stipe of a mature thallus.



TEXT-FIG. 3. Penetration of hyphae from the disc of *Fucus vesiculosus* into a woody substrate. × approx. 420.

The disc of a well-grown plant then, consists of a mass of interweaving hyphae covered over by the pseudo-cortex. The hyphae on the disc are generally about twice the diameter of those in the midrib of the thallus. As a result of the tortuous course which they pursue in the disc, the hyphal cells are often irregular in form and in the diameter of the cell cavity. In the disc the interweaving hyphae exhibit frequent branching, while elsewhere in the thallus the hyphae seldom branch. Branching of the hyphae in the disc is frequently followed by evection.

Text-fig. 3 shows a section taken towards the outer limit of a disc of a well-developed thallus removed from a wooden breakwater. Hyphae from the thallus have penetrated into the xylem tracheides of the wood (Pl. XIV, B) and nearest to the thallus this hyphal penetration has resulted in the breakdown of the walls of the tracheides beyond the surface layer already eroded by the sea. In the older part of the disc this has resulted in the inclusion of small pieces of lignified cell-walls amongst the hyphae.

This penetration of hyphae from the attaching disc of *Fucus vesiculosus* into a wooden breakwater is similar to the penetration observed by Grubb (1923) from the attaching organs of *Porphyra umbilicalis*. Penetration into the substrate accounts for the extreme difficulty in removing a complete attaching disc from the substrate, for the disc always brings away with it the surface layers of rock or substrate to which it is adhering. The actual penetration of hyphae into the substrate may be essential for the attachment of a thallus of *Fucus vesiculosus*, and this would account for the absence of this species from certain kinds of rock, e.g. smooth granitic rocks weathered on an exposed shore. It is true that in small cracks and fissures in the smooth surface numerous sporelings may be found, but few if any develop further.

ATTACHING DISCS FROM A SHELTERED HABITAT

While collecting plants of *Fucus vesiculosus* from a sheltered bay in Loch Melfort (Argyllshire) during September 1946 it was observed that complete attaching discs were easily removed from the substrate of granitic boulders. Numerous rudiments of adventitious branches had developed from the undersurfaces of these discs so that only small areas were adhering to the substrate. It is common to find proliferations arising from the upper surfaces of the attaching discs of this species. But in these discs there has been loss of adhesion followed by proliferation from what is normally the adhering surfaces of the discs. The occurrence of this in a sheltered habitat where there is little pulling force of the thallus to resist is interesting to compare with the complete absence of attaching discs in salt-marsh forms of *Fucus vesiculosus*.

ATTACHING DISCS FROM EPIPHYTIC PLANTS

Thalli of the Fucaceae are often covered with epiphytic growths of filamentous brown algae, but in the literature there is no reference to the epiphytic habit of *Fucus vesiculosus* itself. Yet while collecting this species off the west coast of Argyllshire, where there is such prolific growth of fucoids, plants can frequently be found growing upon *Ascophyllum nodosum* and occasionally upon *Fucus serratus* and *Fucus vesiculosus*. The thalli of both *Fucus vesiculosus* and of the host show normal development.

Sections taken through the disc of a sporeling of *Fucus* which had developed on a thallus of *Fucus vesiculosus*, about 3 in. from the apex of a vegetative branch, showed fine hyphae descending from the sporeling and penetrating well into the tissues of the host. The hyphae pushed in between the medullary cells of the host, but they did not enter any of the host cells. The cortex of the sporeling had established continuity with the cortex of the host, and the absence of any signs of cortex of the host immediately beneath the disc of the sporeling suggested that the original entry of the sporeling rhizoids was through a wound in the surface of the thallus of the host.

The photomicrographs in Pl. XIV, C and D, are of sections taken through the attaching disc of a mature thallus of *Fucus vesiculosus* which was growing upon *Ascophyllum nodosum*. The disc above the tissues of the host showed the normal structure of a disc developing upon a rock substrate, but the advancing margins of the disc had stimulated the meristoderm of *Ascophyllum* to great activity. Large and lightly staining cells were cut off by the meristoderm of *Ascophyllum* as if to form more cortex. But descending hyphae from the disc of *Fucus* penetrated between these cells, and so cortical filaments of *Ascophyllum* became included in the region of the disc. The cells of these cortical filaments grew much larger and were more rounded in form than were the original cortical cells of *Ascophyllum*. The latter provided a boundary beyond which hyphae from the disc of *Fucus* were unable to penetrate. In the centre of the disc there was close union between the cortical filaments of *Ascophyllum* and the interweaving hyphae of the disc of *Fucus*, and this association of the tissues formed a secure attachment for the thallus of *Fucus vesiculosus*. There was no penetration of hyphae into the cells of *Ascophyllum*, and no suggestion of parasitism as described by Batten (1923) for *Polysiphonia fastigiata* growing upon *Ascophyllum*. Thus the presence of these attaching discs of *Fucus vesiculosus* upon other members of the Fucaceae are examples of epiphytism, arising in a habitat where there is prolific growth of fucoids and great competition for the available substrate.

This investigation was carried out in the Department of Botany, Westfield College, during the tenure of a Postgraduate Research Studentship. I wish to express my sincere thanks to Dr. E. M. Delf for helpful criticism.

SUMMARY

1. The development of attaching organs in *Fucus vesiculosus* has been studied.

2. Attachment of the germinating zygote to the substrate is secured by a primary rhizoid, surrounded by a mucilaginous envelope. During later development attachment of the sporeling is aided by hyphae growing from the tissues of the thallus.

3. Sporelings raised on a substrate of pith show that the rhizoids and hyphae of the sporeling penetrate the interstices of the substrate.

4. Hyphae from the main thallus also penetrate into the attaching organ, push their way to the upper surface, and there segment, forming a pseudo-

cortex over the upper surface of the attaching disc, from which parallel filaments can be traced into the centre of the disc.

5. Discs collected from a wooden breakwater show numerous hyphae penetrating down into the substrate.

6. Discs of mature thalli collected from a very sheltered habitat showed the development of numerous rudimentary adventitious branches on the under-surface adjacent to the substrate.

7. Discs of *Fucus vesiculosus* growing upon thalli of *Fucus vesiculosus* and *Ascophyllum nodosum* are recorded from the west coast of Argyllshire. In the former example, hyphae penetrated between the cells of the host, while in the latter the meristoderm of the host was stimulated to great activity and the filaments of cortical cells produced became included in the disc of *Fucus*. In neither case were cells of the host entered, so that these are examples of true epiphytism of *Fucus vesiculosus*, arising in a habitat where there is great competition for the available substrate.

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DESCRIPTION OF PLATE XIV

A. Photomicrograph of part of R.L.S. through disc of mature plant of *Fucus vesiculosus*, showing parallel hyphal filaments. × 40.

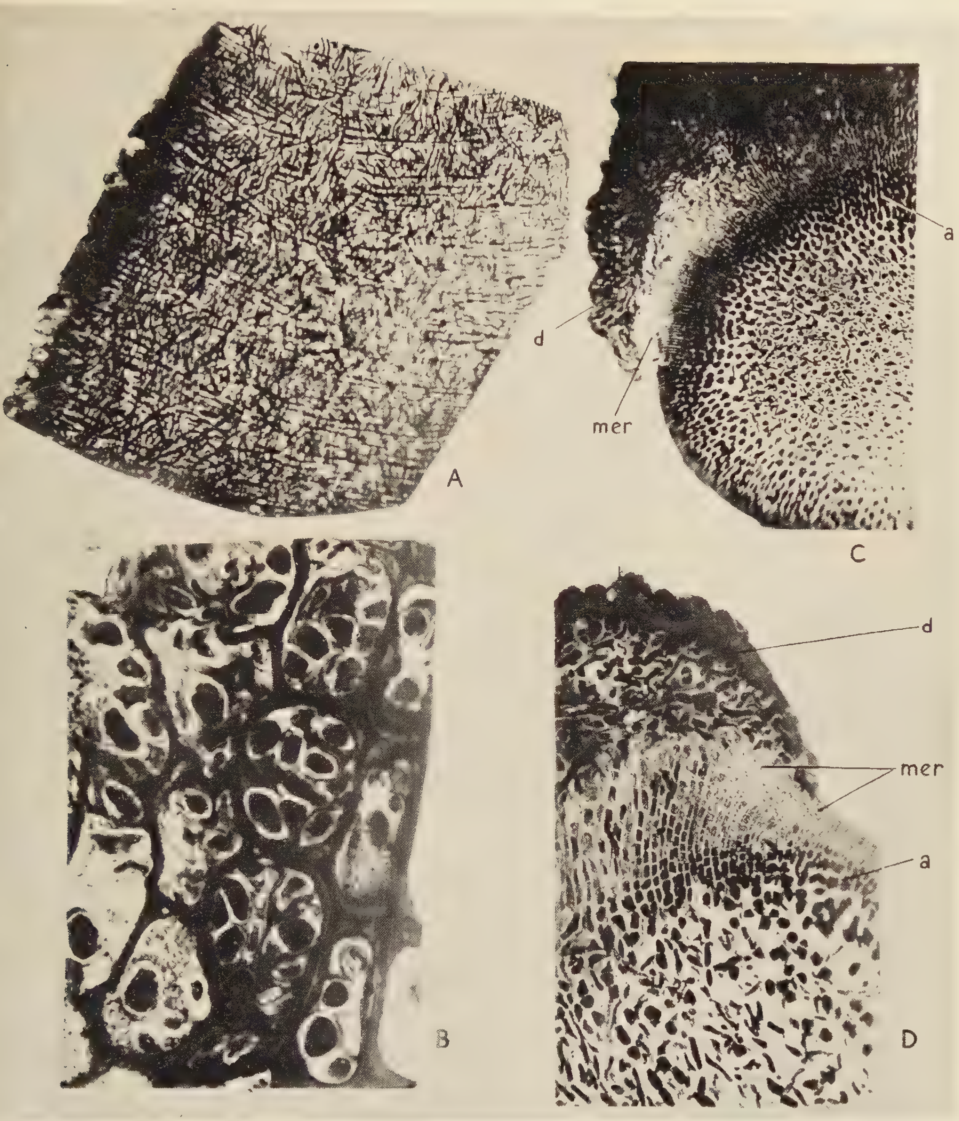
B. Photomicrograph of T.S. through a substrate of wood beneath a disc of *F. vesiculosus*, showing xylem tracheids filled with hyphae. × 380.

C and D. Photomicrographs of discs of *F. vesiculosus* epiphytic upon *Ascophyllum nodosum*, showing stimulation of the meristoderm (*mer*) to great activity.

C. Ascophyllum in transverse section. × 40.

D. Ascophyllum in longitudinal section. × 82.

d = disc of Fucus. *a* = cortex of Ascophyllum. *mer* = meristoderm.



B. L. MOSS

A Quantitative Study of Shoot Development in *Vicia faba*

I. The Xylem of the Plumule

BY

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(*University of Nottingham*)

With six Figures in the Text

ALTHOUGH the development of the vegetative shoot in dicotyledons and particularly the development of its vascular system have long been the subjects of intensive study there is still a surprising lack of quantitative data. Most of the existing morphological literature, even of the most recent, is written in general descriptive terms, and when measurements are given at all they play quite a minor role in the discussion. Apart from the biometrical work on leaf-shape recently reviewed by Ashby (1948) the intensive application of quantitative methods is found mainly in physiological studies such as the investigation by Goodall (1949) of the growth of the seedling of *Theobroma*. Understandably, though none the less regrettably, the writings of physiologists often display a combination of accurate measurement and refined statistical treatment with morphological analysis of the crudest description. This weakness is apparent both in the investigations on hormones as activators of cambial growth (e.g. Snow, 1935) and in the accounts of the anatomical changes associated with photoperiodic phenomena (e.g. Wilton, 1938; Wilton and Roberts, 1936).

The present investigation was originally undertaken in the hope of elucidating by defoliation experiments the part played by the leaf in the development of the trace bundles connected with it. Although it has been very generally assumed by anatomists that the development of trace bundles is in some way under the control of the leaf which they serve, very little experimental work has been done which can be regarded as supporting this interpretation. Not only has it not always been appreciated that the results of decapitation experiments, important though they may be in other connexions, can have little bearing on this particular problem, but most of the experiments which might have contributed to this branch of anatomy have been carried out by physiologists who have not distinguished between leaf traces and other bundles.

A study of the normal course of development of the selected material is obviously an indispensable preliminary to the actual defoliation experiments. The present paper therefore deals with the normal growth of the primary vascular system of the plumule of *Vicia faba*. Only a few of the lower internodes are described, and the account is not carried beyond the point at

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which the interfascicular cambium begins to contribute to the xylem. It is intended that later papers in this series should describe the development of axillary branches and the effects of defoliation and other experimental treatments.

MATERIAL AND TECHNIQUE

Seeds of *Vicia faba* 'Claudia' were sown singly in 3-in. pots in a heated greenhouse. In order to bring about the strongest possible growth of the plumule all lateral shoots were removed as soon as they began to grow. The material for anatomical study was initially selected with some care.

The shoots of *V. faba* bear leaves alternately in two rows, the first and second leaves of the plumule being reduced to small scales which are presumably of little importance as photosynthetic organs. It seems that the conditions in the first and second internodes must therefore be different from those in the succeeding internodes, and the present study begins with the third internode, which is the first one to bear a normal foliage leaf. The third leaf and those immediately following it normally have two leaflets each, together with a small terminal process generally regarded as a vestigial tendril. The third leaf fairly frequently has a terminal leaflet in place of the tendril rudiment, but the occurrence of a terminal leaflet on any leaf other than the third is apparently very uncommon. Plants exhibiting these features have been excluded from the material studied. The upper leaves have more than two leaflets, a condition which generally appears first in the ninth or tenth leaf of the plumule. This change, which is followed almost at once by the initiation of flower-buds, may reasonably be regarded as marking the end of the seedling stage, and the present study is concerned mainly with the internodes which are associated with bifoliolate leaves.

A transverse section of a plumular internode is shown in Fig. 1. The vascular bundles in this section fall into three categories. Firstly there are the two cortical bundles which give off at each node branches which enter the leaf as lateral traces, the cortical strands being replenished by bundles which join them from the central cylinder of the stem. The cortical bundles are of a decidedly anomalous nature, being neither leaf traces nor typical stem bundles, and are therefore omitted from the subsequent discussion. Secondly there are the two large and conspicuous bundles ${}_nM_n$ and ${}_nM_{n+1}$, situated on the diameter at right angles to that which includes the cortical bundles. One of these bundles is the median trace of the next leaf above the plane of the section, the other is the median trace of the leaf above that. Fortunately it is always possible to distinguish between them, since the cortical bundles are always placed so that their xylem inclines towards the median trace of the first leaf above the plane of section. Lastly there are the remaining bundles of the central cylinder. None of these bundles are leaf traces, in the strict sense of the term, but all of them are ultimately connected with the traces of higher leaves, either with the median traces directly or with the lateral traces by way of the cortical bundles.

In order to obtain numerical results it is necessary to find some way of measuring the degree of development which a particular vascular bundle has reached. The number of methods which are available for this purpose is limited. Almost the only practicable possibilities are the counting of cells and the measurement of areas. The method adopted, after a few preliminary trials, was to count the number of fully lignified and apparently functional cells in the primary xylem as seen in transverse section. This procedure has the advantage, as compared with a measurement of area, of taking into account the differentiation of the tissue as well as its bulk, besides avoiding the difficulties arising from the variable and irregular shapes of the bundles. It is, however, very far from being an ideal form of measurement. The nature of the cells counted is left in doubt. In the younger bundles they are probably all vessels, but in the older bundles other types of element are certainly included. Also, owing to the obliteration of protoxylem elements which can then no longer be counted, there is at least a possibility that at some stage in the development of the bundle there may actually be a decrease in the cell-count. Fortunately this last objection appears to be of theoretical importance only.

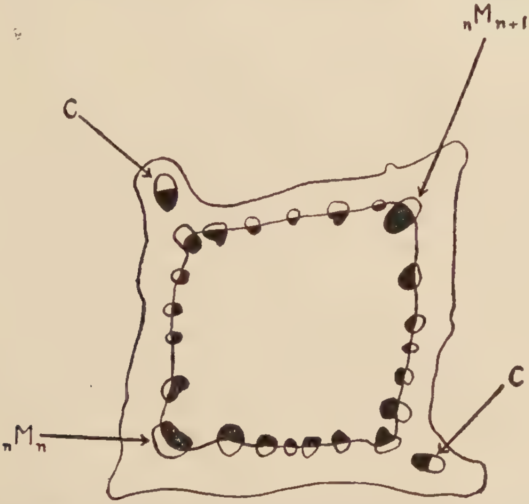


FIG. 1. Transverse section of a plumular internode of *Vicia faba*. $_nM_n$, median trace of first leaf above the plane of section; $_nM_{n+1}$, median trace of second leaf above the plane of section; *CC*, cortical bundles.

It will be convenient to have a standardized system of symbols for the various cell-counts which are to be discussed. Taking a section of the nth internode, we may write $_nM_n$ for the count of the median trace of the nth leaf, and $_nM_{n+1}$ for that of the median trace of the $(n+1)$th leaf. The remaining bundles of the central cylinder cannot be dealt with individually, since they are extremely variable in number and arrangement, but we shall have occasion to consider their total cell-count, which will be denoted by T_n.

Throughout the investigation the sections have been cut freehand from about the middle of the internode and stained with phloroglucin and hydrochloric acid, the cells being counted under a $\frac{1}{6}$-in. objective. In leaf traces the cells were generally counted individually, but *T* is often so large that an actual count is hardly practicable. In such cases an estimate has been made by counting the cells in one of the larger bundles and then judging by inspection the factor by which this figure is to be multiplied to give the value of *T*.

THE MEASUREMENT OF AGE

One of the principal difficulties in all work of this kind arises from the lack of any natural scale by which to assess the age of the material. It is utterly useless to group the observations according to the chronological age of the plants. Not only is the rate of growth dependent on temperature and other conditions to such a degree as to make it impossible to compare two series of observations made at different times, but the variability of individual plants is so great that plants of the same chronological age may have reached very different stages of morphological differentiation, while plants which are structurally similar may be of quite different chronological ages. These differences are brought about by unavoidable unevenness in speed of germination, slight differences in the depth at which the seed is sown, and so on. What is needed is some criterion by which to judge the 'physiological age' of the material, in the sense in which this term is used by Ashby and others. In the present investigation this problem arises in two distinct forms. It is necessary to have not only a measure of the physiological age of the plant as a whole, but also a means of expressing the physiological age of a single internode, since the internodes of a plant are obviously all of different ages.

So far as the physiological age of a single internode is concerned, it has for the present purpose been assumed that the observed value of *T* is a sufficiently accurate measure. This assumption is justified by the fact that *T* represents the sum of the cell-counts of all the bundles serving the upper part of the shoot, and therefore increases as the internode in which *T* is measured becomes farther and farther removed from the apex. So long as the observations are confined to the counting of cells there is really no other criterion available, and there is no obvious reason why estimates obtained by other methods should be more truly representative.

The physiological age of a plant as a whole has been determined by using the time scale provided by the formation of successive leaves at the shoot apex. This scale, the interval of which is the plastochrone, is generally accepted as the appropriate scale for studies on shoot differentiation. In applying it to the purposes of the present investigation the procedure has been to count up the shoot to the highest leaf which is fully unfolded or shows at the most a slight rolling of the extreme edges. The serial number of the next leaf is taken as the age of the plant in plastochrones. Thus, if the age of a plant is recorded as 8, this implies that the plant has five fully expanded foliage leaves (numbers 3–7 inclusive), leaf 8 being in an immature condition and not yet fully open. This scale of time is obviously a crude one, since there is no way of estimating fractions of a plastochrone. Attempts to distinguish finer subdivisions in the unfolding of a leaf have not so far been very successful. A mathematical treatment will be given elsewhere by which this difficulty may perhaps eventually be circumvented in certain cases. Unfortunately the observations which are described here do not fulfil the required conditions, and no simple mathematical treatment can at present be applied to the bean plumule. In view of its lack of precision the plastochrone scale has been used as little as possible, and the discussion is mainly based on the use of T as an index of the physiological age of single internodes.

SOURCES OF ERROR

It is evident that the cell-counts are themselves subject to errors of several kinds. Apart from mechanical slips such as counting the same cell twice or dropping one of the groups of twenty in which the counting was carried out, there is considerable room for errors of judgement in deciding which cells are to be included in the count and which are to be excluded on grounds either of immaturity or of obliteration. As, however, all the cell counts have been carried out by the same observer working under standard conditions, there is no reason to suppose that these errors have any important systematic component. The same may be said of the procedure for estimating the larger values of T. Although individual readings may be in error, the average of several observations is probably fairly reliable.

Another possible source of error is the variation of cell-numbers along the length of an internode. Nothing is certainly known concerning the existence or magnitude of such variations, and even if one had a very full knowledge of them it is difficult to see how any correction could be made, since no method is available for finding equivalent points in internodes of different lengths. The only precaution which it has been possible to take is the obvious one of cutting all the sections from the middle portions of the internodes, so as to avoid any disturbances associated with the nodes.

A more subtle kind of error arises from the use of T as an index of physiological age. It is obvious that of two corresponding internodes belonging to different plants, one may at every stage of its development yield a higher cell-count than the other, owing to an inherent difference in vigour between the two individuals. But as the cell-count is used as a measure of age, the more vigorous plant will, quite wrongly, be grouped with physiologically older plants than the less vigorous one. Variations due to age are thus inextricably confused with those due to inherent differences in vigour. The consequences of this confusion cannot be foreseen, but it is probable that no simple statistical treatment of the observations can be strictly valid.

GRAPHICAL METHODS

The variability of the material is so great that the direct plotting of single observations results in the points of a graph being scattered over a considerable area. The probable form of each graph could, of course, be determined by calculating a multinomial regression equation. In view, however, of the uncertainties arising from the use of T as a measure of physiological age, it seems likely that the appearance of accuracy resulting from the calculation of regression coefficients to several significant figures would be dangerously misleading, while the magnitudes of the coefficients would not in themselves be of any particular interest. On the other hand, the use of 'eye-fitted' curves introduces a subjective element which is highly objectionable. A mechanical method of curve-smoothing has therefore been employed. The data are arranged in numerical order of the independent variable, and the resulting table is divided into sets of five observations. At the end of a table three or four observations are allowed to form a set, while if there are only one or two observations left they are added to the preceding set. Both variables are then averaged for each set, so that each point of the graph, except perhaps the last one, is based upon five observations. This operation is absolutely free from any personal bias, its principle effect being a slight flattening of all the curvatures of a graph, bringing it artificially nearer to a straight line than is really the case. The same procedure has been used for all the graphs except those in Fig. 2, where the scale of abscissae is naturally discontinuous.

In plotting graphs of M against T, both variables have been plotted logarithmically. This method has no particular theoretical significance, but is employed simply as a convenient means of magnifying the earlier part of a curve at the expense of the remainder.

THE BEHAVIOUR OF T

The continuous curves of Fig. 2 illustrate the way in which T changes with the increasing plastochrone-age of the plant. It is clear that in each internode T at first increases very slowly, but afterwards at an increasing rate. It is perhaps not immediately obvious that the behaviour of T is significantly different for every internode, but a careful examination reveals two important trends. The upper broken line shows for each internode the value of T in that internode during the unfolding of the associated leaf. In order to show this curve on a reasonable scale all ordinates have been multiplied by 20. This curve shows a rapid fall from the third internode to the fifth, followed by a rather more gradual rise. During the unfolding of the third leaf there are about 22 vessels serving the shoot apex, whereas during the unfolding of the fifth there are only about 6. The latter figure seems astonishingly small, since these 6 vessels, together with an unknown but certainly quite small number in the cortical bundles, must supply the whole of the necessary water for the apical meristem and for the associated leaf-primordia, some of which are of considerable size. It is obvious that this attenuation of the vascular supply to the apical region cannot continue indefinitely, and in fact the graph shows a rise in the number of vessels serving the meristem until at the unfolding of the ninth leaf there are 16.

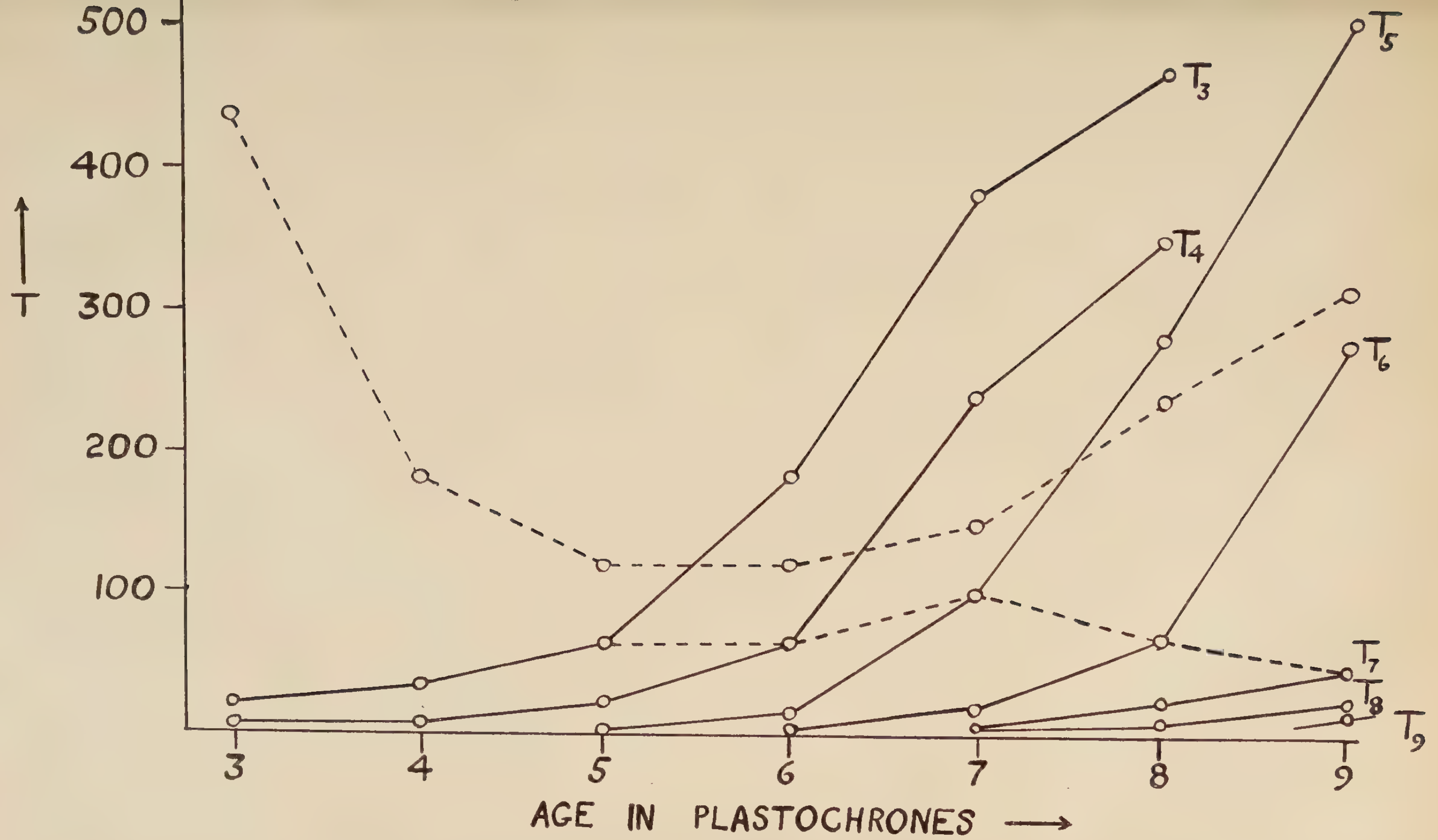


FIG. 2. Continuous lines are graphs of T against plastochrone-age of the plant. Upper broken line shows the value of T for each internode at the time when the corresponding leaf unfolds (in drawing this curve ordinates have been multiplied by 20). Lower broken line shows value of T for each internode at the time when that internode ceases to elongate.

Less conspicuous, but quite as important, is the change in the gradient of the curves of T as one progresses from the third internode towards the upper part of the shoot. The graph of T_4 is a little steeper than that of T_3, and that of T_5 is steeper still, but the internodes above the fifth show a falling-off in the steepness of the curve. This implies that the development of the fourth and fifth internodes follows more closely upon that of their predecessors than is the case for later internodes. This effect may be demonstrated more clearly by plotting the values of T for each internode not against the age of the plant

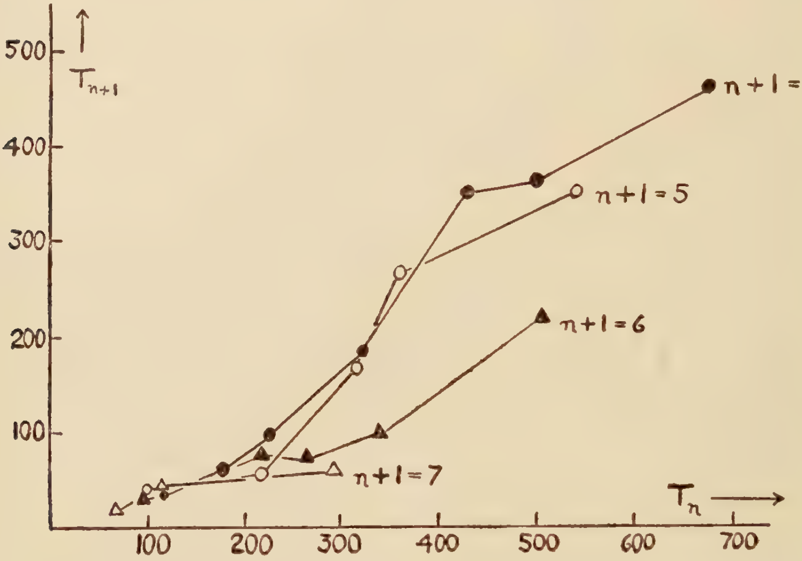


FIG. 3. Graphs of T_{n+1} against T_n.

as a whole, but against the value of T for the preceding internode. When this is done it is found that each internode yields a curve of characteristic form. In Fig. 3 are shown the curves obtained by plotting T_{n+1} against T_n for the lower internodes of the plumule. These curves show very plainly that the development of the fourth internode follows upon that of the third more closely than that of the fifth does upon the development of the fourth, and so on. Another point brought out in a striking manner by these curves is the fact that these differences between successive internodes become apparent mainly in the later stages of development. During the early stages, when T is less than about 50, the development of every internode is related to that of its predecessor in approximately the same manner.

It is a matter of some interest to determine what is the value of T at the time when the internode ceases to increase in length. For this purpose a considerable number of rapidly growing plants of various ages have been marked with indian ink by means of a simple device which makes two transverse lines about 3 mm. apart on each internode to which it is applied. The plastochrone-age of the plant is recorded at the time of marking. After 2 or 3 days the marks upon the stems are compared with the marker that made them. Although there is a certain amount of variation, most of the observations agree in that the longitudinal growth of the nth internode is found to have ceased during the unfolding of the leaf $(n+2)$. The relevant values of T are indicated by the lower broken line in Fig. 2. The various internodes yield values lying approximately in the range 50–100, with an average of about 70. Owing to the unsatisfactory nature of the plastochrone time scale the more exact determination of the critical value of T would be an undertaking of some difficulty. There is no satisfactory evidence for any variation in the value from internode to internode. It is, however, rather striking that the value of T when elongation ceases does not markedly differ from that at which the curves given in Fig. 3 first begin to separate.

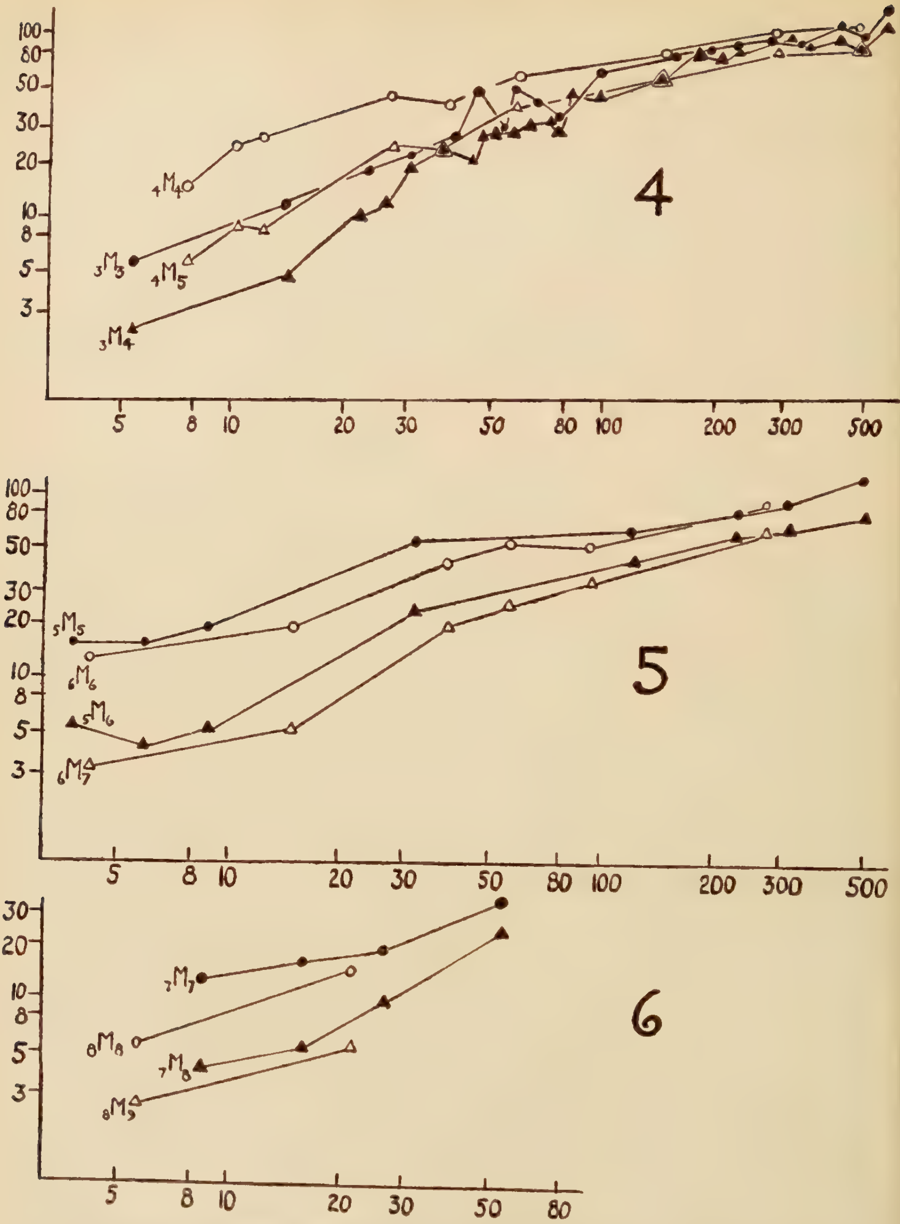
THE BEHAVIOUR OF M

In order to avoid the use of the inaccurate plastochrone scale, the variation of M has been studied with reference to the corresponding values of T. The phenomena are best represented by plotting graphs of ${}_nM_n$ and ${}_nM_{n+1}$ against T_n. For purposes of comparison it would be convenient to have all the graphs for the internodes investigated plotted on the same axes. Unfortunately this is hardly practicable, owing to the crowding which would result. The curves have therefore been divided into three groups. Fig. 4 gives the graphs for the third and fourth internodes, Fig. 5 those for the fifth and sixth, and Fig. 6 those for the seventh and eighth.

A comparison of all these curves shows an important trend as one passes up the shoot from the third internode to the eighth. From an inspection of Fig. 4 it will be clear that the curves for the fourth internode run considerably above the corresponding ones for the third internode. Although the reader will only be able to verify the fact by making measurements, the curves for the fifth internode are almost exactly of the same form as those for the fourth. It will be found from Figs. 5 and 6 that there is then a steady fall of the curves with each successive internode, until internode 8 is reached, which yields curves almost identical with those for internode 3.

These results may be expressed in either of two ways. Either it may be said that the development of the leaf traces is relatively tardy in the third internode, is quicker in the fourth and fifth, and becomes gradually slower in the subsequent internodes; or it may be said that the development of the other bundles in the stem is hastened in the third internode, relatively delayed in the fourth and fifth, and speeded up again in the internodes above. The form of expression adopted will depend on which time-scale is chosen as the more fundamental—the one based on the development of the leaf trace, or the one based on the development of the internode as a whole.

It is obviously a matter of some interest to determine what is the number of vessels in the median trace of a leaf at the time when that leaf is unfolding. This can easily be done by taking from Fig. 2 the values of T indicated by the



FIGS. 4–6. Graphs of M against T, both variables plotted logarithmically. Fig. 4, internodes 3 and 4; Fig. 5, internodes 5 and 6; Fig. 6, internodes 7 and 8.

upper broken line and reading off the corresponding values of M from the curves of $_nM_n$ given in Figs. 4–6. (In a few instances a slight extrapolation is required.) This method will, of course, give the number of vessels in the upper part of the trace. To determine the number in that portion of it which lies in the lower internode of the two which it traverses we must proceed in a rather different manner. Reading off from the graphs of Fig. 2 the value of T_n at the time when the leaf $(n+1)$ is unfolding, the corresponding value of $_nM_{n+1}$ is found from the appropriate curve of Figs. 4–6. The results of these investigations are set out in Table I, and show that there is, as one traverses the stem upwards, a steady decline in the number of vessels in the median trace of the leaf at the time when the leaf unfolds. This decline is more marked in the lower part of the leaf trace than in the upper part. The numbers of vessels are all surprisingly small. The leaf as it unfolds has only some 10 or 15 vessels in the upper part of its median trace and a number not exactly known but probably no greater in its two lateral traces. It would seem on general grounds that the decline in the number of vessels serving the leaf at the time of its unfolding cannot continue indefinitely in the higher part of the stem.

TABLE I

Values of M_n at the Time of Unfolding of Leaf n

n	$_{n-1}M_n$	$_nM_n$
3	—	16·6
4	21·8	16·6
5	20·8	14·5
6	11·5	13·8
7	13·8	11·7
8	10·0	10·9
9	6·3	—

There is yet another way of studying the variation of M. It has already been shown that the upper and lower part of the same median trace may behave differently, and it is obviously of some interest to find how the development of the upper part is related to that of the lower. This would be most conveniently done by plotting graphs of $_{n+1}M_{n+1}$ against $_nM_{n+1}$. Unfortunately the graphs obtained from the data of the present investigation are too irregular to be worth publishing. Instead there are set out in Table II the mean values of the fraction $_{n+1}M_{n+1}/_nM_{n+1}$ for the traces of leaves 4–8 inclusive, within the range investigated. It is clear from these figures that in the median trace of the fourth leaf the development of the upper part keeps pace with that of the lower, but that in the traces of higher leaves the upper part lags more and more behind the lower portion of the same trace. There is a decided tendency for the fraction to be larger in the earlier stages of development, and the slight

TABLE II

$n+1$	$_{n+1}M_{n+1}/_nM_{n+1}$
4	1·016
5	0·976
6	0·859
7	0·654
8	0·720

rise in the value for the trace of leaf 8 is probably to be attributed to this effect, since this trace has only been studied in its younger stages.

DISCUSSION

Sufficient data have been obtained to make it possible to give a fairly detailed account of the development of the xylem of each internode of the bean plumule within the range of this survey. Considering first the general development of the internode itself, without reference to the leaf-traces which traverse it, we see that in the earlier part of its history, up to a point considerably beyond the stage at which the associated leaf unfolds, the internode develops in a manner which bears a constant relationship to the development of the internode below. At about the time when stem elongation ceases, the behaviour of the internode begins to depend on its position in the stem, development being more rapid, relative to that of the preceding internode, in the lower internodes of the plumule. It is tempting to suppose that the marked stimulation shown by the lower internodes at this point may be due to the presence of reserve substances in the cotyledons. It is intended at a later date to carry out experiments designed to test this hypothesis.

Regarding the development of the leaf traces, it is particularly interesting to find that there is no fixed relationship between the development of a trace bundle and that of the internode in which it is situated. Furthermore, the degree of trace development corresponding to the unfolding of the leaf is different for every internode. These facts serve to emphasize the unsatisfactory nature of the plastochrone time scale. It seems perfectly clear that the unfolding of the leaf, unlike the cessation of stem elongation, does not mark any definite epoch in the development of the internode.

The relationship between the upper and lower parts of the same median trace has a direct bearing on the question of the control by the leaf of the development of its traces. It is quite clear that for the leaves above the fourth the lower part of the trace is, at least in the later stages, always in advance of the upper part. This is the reverse of what would be expected if development were stimulated by any influence travelling downwards from the leaf. Whatever may be true of the early stages, the development of the trace after the unfolding of the leaf is markedly acropetal.

The results of this investigation also raise some wider issues. It has been shown that the developing plumule undergoes a complex series of structural changes, each internode being different in various ways from the ones above and below. From the very nature of some of these changes, notably those expressed by the graphs of Fig. 3 and the figures of Tables I and II, it is impossible that they can continue indefinitely in the same direction. There must be a lower limit to the number of vessels serving a leaf at the time when it unfolds, just as there must be a limit to the amount by which the development of an internode can lag behind that of the preceding one. It follows that at some time during the life of the individual these changes must be arrested, possibly reversed. Other measurements undergo a cycle of changes, as shown by the upper broken line in Fig. 2 and by the rise and fall of the curves of M against T in Figs. 4–6. In respect of these characters the higher internodes return approximately to the condition found in the lower ones.

It is impossible to escape the conclusion that all these changes are in some way the result of the alteration in circumstances associated with the transition from a seedling depending on the reserves in the cotyledons to a mature plant independent of such reserves. If this is so, it is to be expected that the changes will not be repeated or indefinitely continued, but that the plant will eventually reach an equilibrium condition in which each internode will be approximately the same as the ones above and below. Even if this expectation is not strictly fulfilled, it is still to be anticipated that there will be some stage at which the influences derived from the seed no longer play any important part in deciding the behaviour of the plumule. The attainment of physiological independence naturally marks an important epoch in the history of the plant and is likely to be associated with characteristic morphological and physiological manifestations in the plumule.

The data at present available give no indication of the attainment by the plumule of an equilibrium condition. There are, however, several reasons for supposing that the period between the ages of 8 and 10 plastochrones must be of special significance in the life of the plumule. During this period all those quantities which have been shown to vary in a cyclic manner regain approximately the values which they had in the third internode. At this time the value of M at the unfolding of the leaf has become so small that it seems hardly possible that it can fall much farther. Of far greater significance, however, is the fact that it is at this time that the relative tardiness of the growth of the upper internodes first begins to operate. An inspection of Fig. 2 will show that T_6 first exceeds 50 when the age of the plant is about $7\frac{1}{2}$ plastochrones. That is to say, at the beginning of the period which we are now considering, the sixth internode has just entered upon that part of its development which is expressed by the characteristic part of the graph of T_6 in Fig. 3. The resulting retardation not only of the sixth internode but also of all those above it must have a profound effect upon the apical region, and in fact growth curves indicate that at this time the rate of increase in height falls off very markedly.

It is therefore of considerable interest that the multifoliolate condition first appears, in most cases, in the eighth, ninth, or tenth leaf, and that the first flower-buds become visible a little later, suggesting that their initiation occurred during this critical phase. It is hoped that it will later be possible to make a more detailed study of plants in this transitional stage of their development.

SUMMARY

1. The development of the primary xylem of the plumule of *Vicia faba* has been studied by counting the numbers of lignified cells visible in transverse sections.

2. During the early stages, while an internode is still elongating, its vascular differentiation is related to that of the internode below in a manner which is essentially the same for all internodes.

3. After an internode ceases to elongate, the further elaboration of its xylem depends on its position in the stem, the lower internodes showing at this stage a stimulation which is not evident in the higher ones.

4. It is tentatively suggested that this stimulation may be due to the presence of reserve substances in the cotyledons.

5. Relative to the general vascular differentiation of the internode, the leaf traces develop more rapidly in the fourth and fifth internodes than in those above or below.

6. The higher the position of a leaf on the stem, the greater is the tendency for the development of the upper part of its trace to lag behind that of the lower part.

7. The time of unfolding of a leaf bears no fixed relationship to the development either of its own traces or of the internode which bears it.

8. The higher a leaf is placed upon the stem, the smaller is the number of vessels in its median trace at the time when the leaf unfolds.

9. The facts of vascular development seem to indicate that the period immediately following the unfolding of the eighth leaf may be of special significance in the life of the plumule.

10. At about this time the first multifoliolate leaves and the first flower-buds appear.

11. It is likely that this is the time at which the plant becomes fully independent of the cotyledonary reserves.

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